

# **Analytical Profiles of Drug Substances**

**Volume 17**

*Edited by*

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New Brunswick, New Jersey

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## PREFACE

Although the official compendia define a drug substance as to identity, purity, strength, and quality, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. Such information is scattered through the scientific literature and the files of pharmaceutical laboratories.

I perceived a need to supplement the official compendial standards of drug substances with a comprehensive review of such information, and seventeen years ago, the first volume of *Analytical Profiles of Drug Substances* was published. That we have been able to publish one volume per year is a tribute to the diligence of the editors to solicit articles and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medical value, and therefore, we have welcomed any articles of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

Klaus Florey

# **AZTREONAM**

**Klaus Florey**

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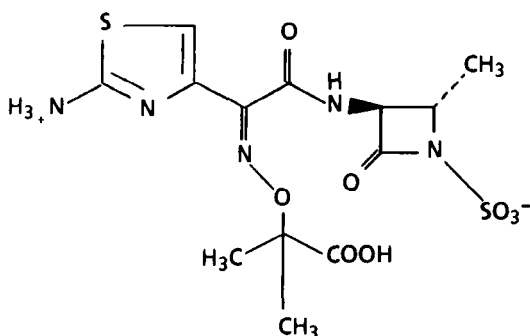
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## 1. Description

### 1.1 Name, Formula and Molecular Weight

Aztreonam, also azthreonam and SQ 26,776 in the early literature.

(1) Propanoic acid, 2-[[[1-(2-amino-4-thiazolyl)-2-[(2-methyl-4-oxo-1-sulfo-3-azetidinyl)amino]-2-oxoethylidene]amino]oxy]-2-methyl-, [2*S*-[2*α*,3*β*]*Z*]]-; (2)*Z*-2-[[[(2-Amino-4-thiazolyl)[[(2*S*,3*S*)-2-methyl-4-oxo-1-sulfo-3-azetidinyl] carbamoyl] methylene]amino]oxy]-2-methylpropionic acid. CAS-78110-38-0. INN; BAN.



$C_{13}H_{17}N_5O_8S_2$

M.W. 435.43

### 1.2 Appearance, Color and Odor

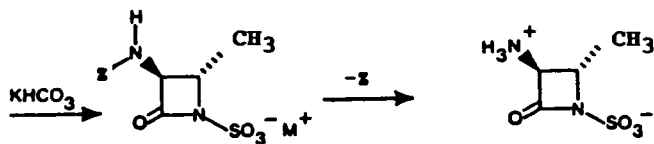
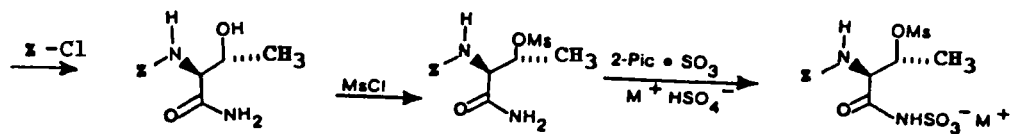
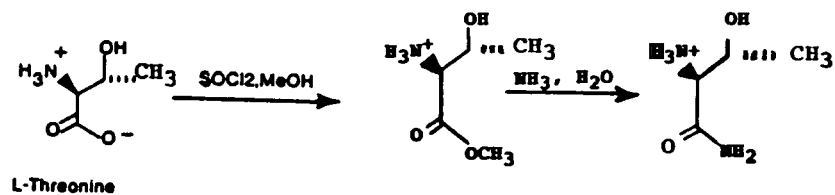
White crystalline, odorless powder.

### 1.3 History

Aztreonam is a synthetic, monocyclic beta-lactam antimicrobial agent, active against gram-negative organism and belonging to a new class of antibiotics, the monobactams. It was developed in the Squibb Laboratories. The events leading to discovery of the monobactams and synthesis of aztreonam have been described <sup>1-6</sup>.

## 2. Synthesis

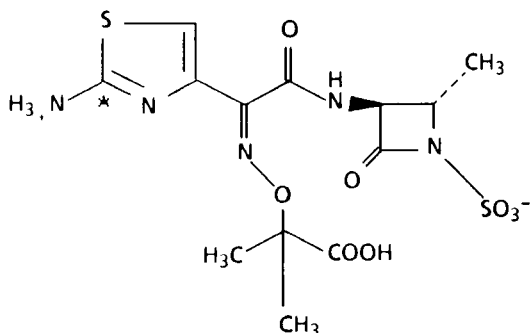
A stereospecific synthesis, starting with L-threonine, of the key nucleus intermediate (2*S*-trans)-3 amino-2-methyl-4-oxo-1-acetidine sulfonic acid) was developed in the Squibb Laboratories <sup>7</sup>. It is presented in Figure 1. By coupling with the side chain, this zwitterion is converted to aztreonam. For synthesis variations, see reference <sup>8</sup>.



**Z = Carbobenzyloxy**

FIGURE 1. Synthetic Pathway to Nucleus Intermediate (Reference 7).

$^{14}\text{C}$ -Aztreonam, labelled as shown, has been prepared<sup>9</sup>.



### 3. Physical Properties

#### 3.1 Infrared Spectra

The infrared spectrum of aztreonam in KBr/MeOH is presented in Figure 2. Infrared spectra (KBr pellets) of the two polymorphic forms  $\alpha$  and  $\beta$  (see 3.11) are presented in Figures 3 and 4<sup>10</sup>.

#### 3.2 NMR Spectra

The 100 MHz proton NMR spectrum of aztreonam in DMSO- $d_6$  (Figure 5) is described in Table 1. The spectrum was obtained on a Varian XL-100-15 NMR spectrometer equipped with a Nicolet TT-100 data system. Instrumental settings: sweep width 1600Hz (quadrature detection); pulse width, 30 $^\circ$ ; pulse delay, 2 sec.; data points, 8192; acquisition time, 2.56 sec.; and probe temperature, 30 $^\circ$  C. The 2.6 Hz coupling constant between the protons of the beta-lactam ring confirms their relative (trans) stereochemistry. The chemical shift of the thiazole proton ( $\delta = 6.82$ ) confirms the Z-configuration for the oxime double bond<sup>11</sup>.

The proton decoupled 15.1 MHz carbon-13 NMR spectrum of aztreonam in DMSO- $d_6$  (Figure 6) is assigned in Table 2, obtained on a JEOL FX-270 spectrometer using a 5mm C/H dual probe. Spectral parameters; sweep width, 15,500 Hz; 500 pulses; 3.5  $\mu$  sec. pulse (45 $^\circ$ ); pulse delay, 1.5 sec.; bilevel decoupling; 16384 data points<sup>11</sup>.

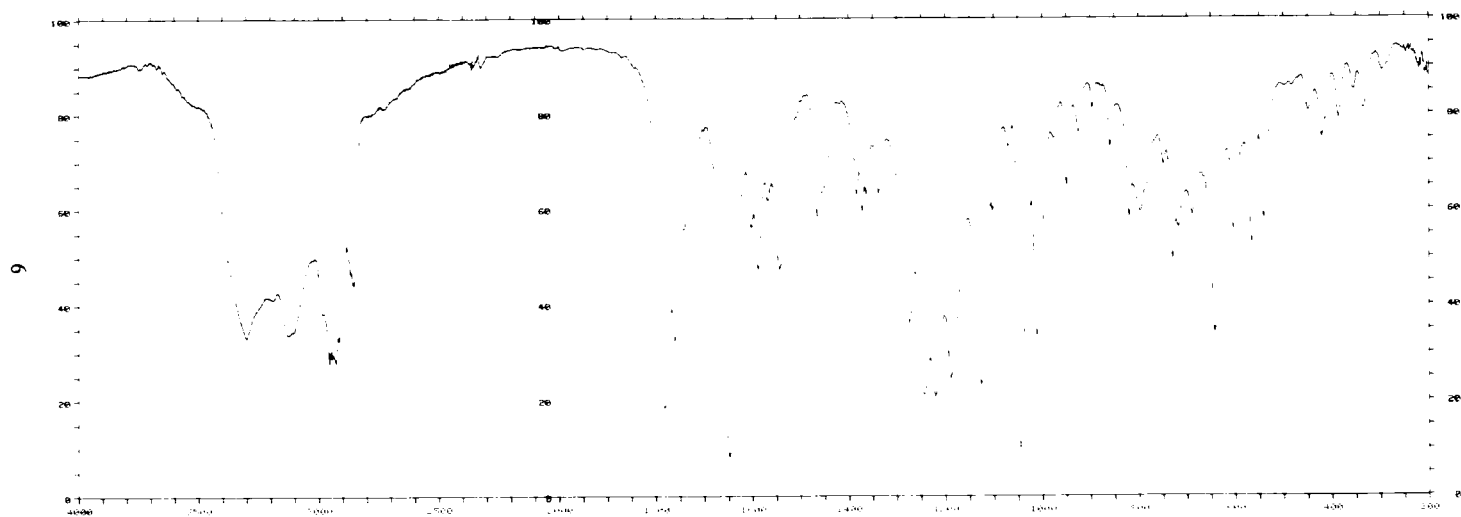


FIGURE 2. I.R. Spectrum of Aztreonam Research Standard AZ028. KBr/MeOH  
Instrument: PE983



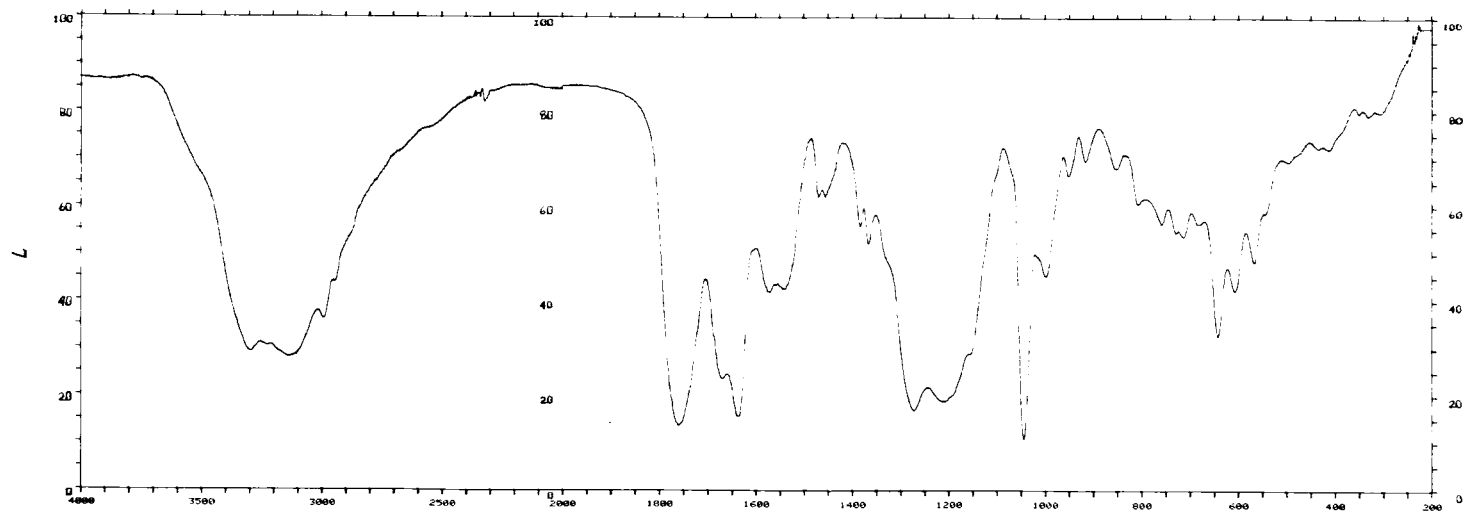
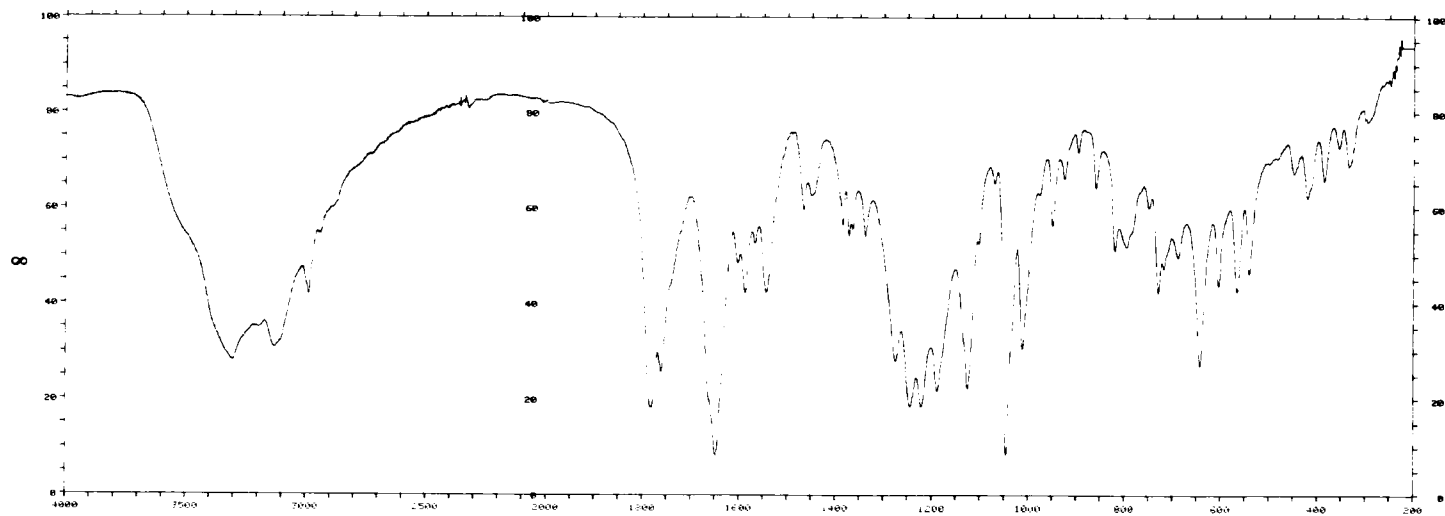


FIGURE 3. I.R. Spectrum of Aztreonam ( $\alpha$  - Form). KBr Pellet.  
Instrument: PE983



**FIGURE 4.** I.R. Spectrum of Aztreonam Research Standard AZ028 ( $\beta$  - Form). KBr Pellet  
Instrument: PE 983

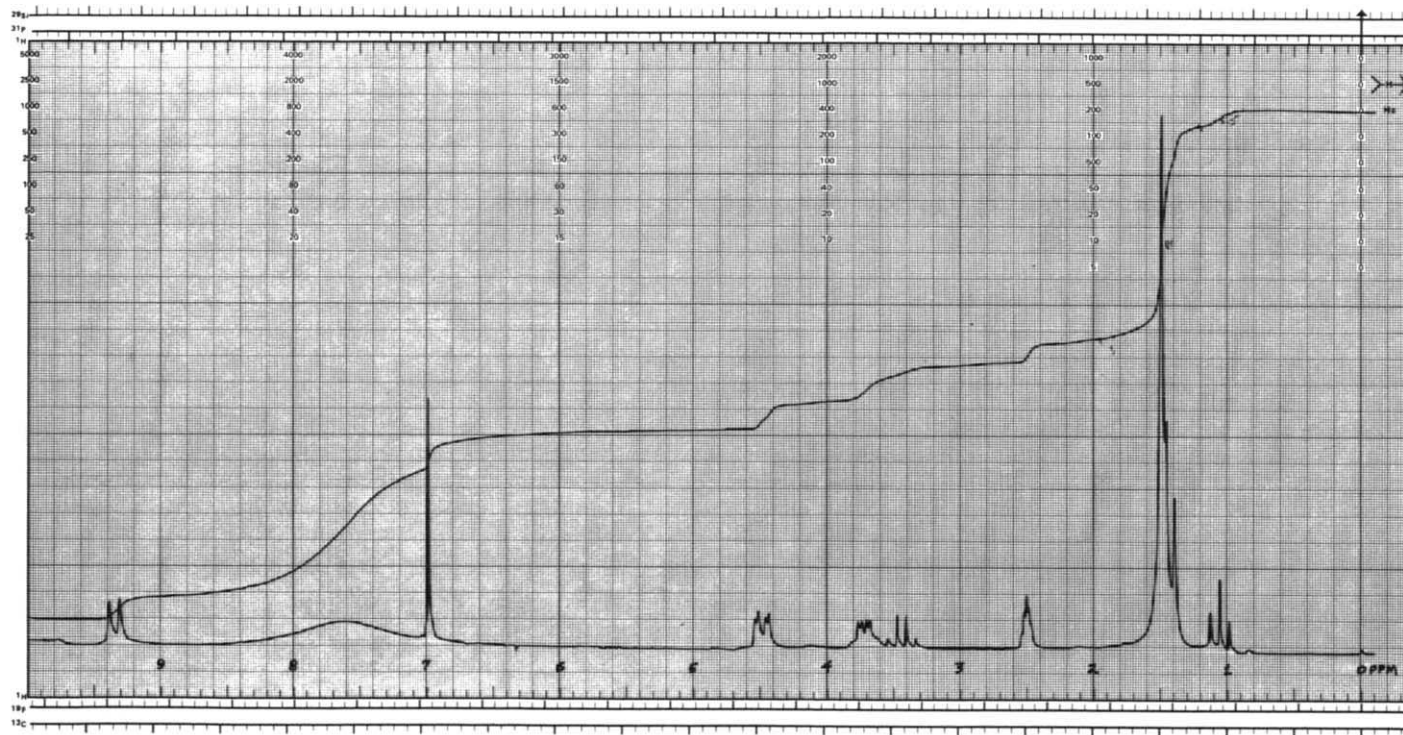


FIGURE 5. Proton NMR Spectrum of Aztreonam Research Standard AZ028  
Instrument: Varian XL-100-15

SQ 26776  
NMR NO. 100515A  
(DMSO)

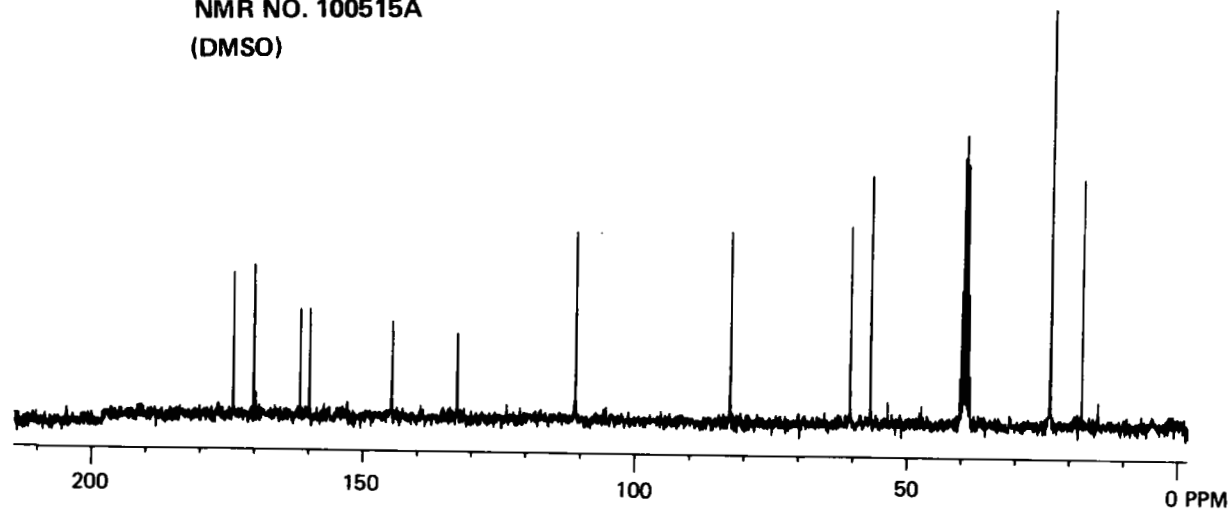


FIGURE 6. Carbon-13 NMR Spectrum of Aztreonam in DMSO at 60° C.  
Instrument: JEOL FX-270

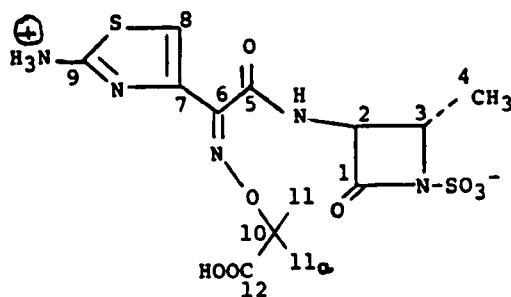
**TABLE 1****100 MHz Proton NMR of Aztreonam in DMSO-d<sub>6</sub>**

Chemical Shift (ppm from TMS)	Number of Protons	Assignment
1.42 d (J = 6.2)	3	<u>CH</u> <sub>3</sub> CH
1.49 s	6	OC( <u>CH</u> <sub>3</sub> ) <sub>2</sub>
3.72 d,q (J = 6.2, 2.6)	1	CH <sub>3</sub> - <u>CH</u> -CH
4.38 d,d (J = 9.0, 2.6)	1	NH- <u>CH</u> -CH
6.86 s	1	Thiazole- <u>H</u>
9.33 d (J = 9.0)	1	N <u>H</u> -CH
~7.6 broad	>4	<u>NH</u> <sub>3</sub> <sup>⊕</sup> , COOH, XH <sub>2</sub> O

The proton and carbon-13 NMR spectra of aztreonam are consistent with the postulated structure<sup>11</sup>.

### 3.3 Mass Spectra

Positive (Figure 7) and negative (Figure 8) mass spectra<sup>12, 13</sup> were obtained on a double-focusing magnetic sector instrument Model ZAB-1F, VG Analytical Ltd., Altrincham, U.K., equipped with a fast atom bombardment source using 4-8 kV xenon neutral atoms. Aztreonam gave a very prominent [M-H]<sup>-</sup> ion (base peak) in the negative ion detection mode and a significant MH<sup>+</sup> ion in the positive ion detection mode. Weak, but perceptible, dimeric ions were also observed.

**TABLE 2****Carbon-13 NMR Data for Aztreonam in DMSO at 60° C.**

<u>Chemical Shift<sup>a</sup></u>	<u>Assignment<sup>b</sup></u>
174.1	12
170.2	9
161.7	1
160.0	5
144.8	6
132.8	7
111.1	8
82.6	10
60.6	2
56.9	3
23.7	11,11a
17.9	4

<sup>a</sup> PPM from TMS with DMSO at 39.5

<sup>b</sup> Assignments based on long range C-H coupling constants.  
Carbon numbering as shown above.

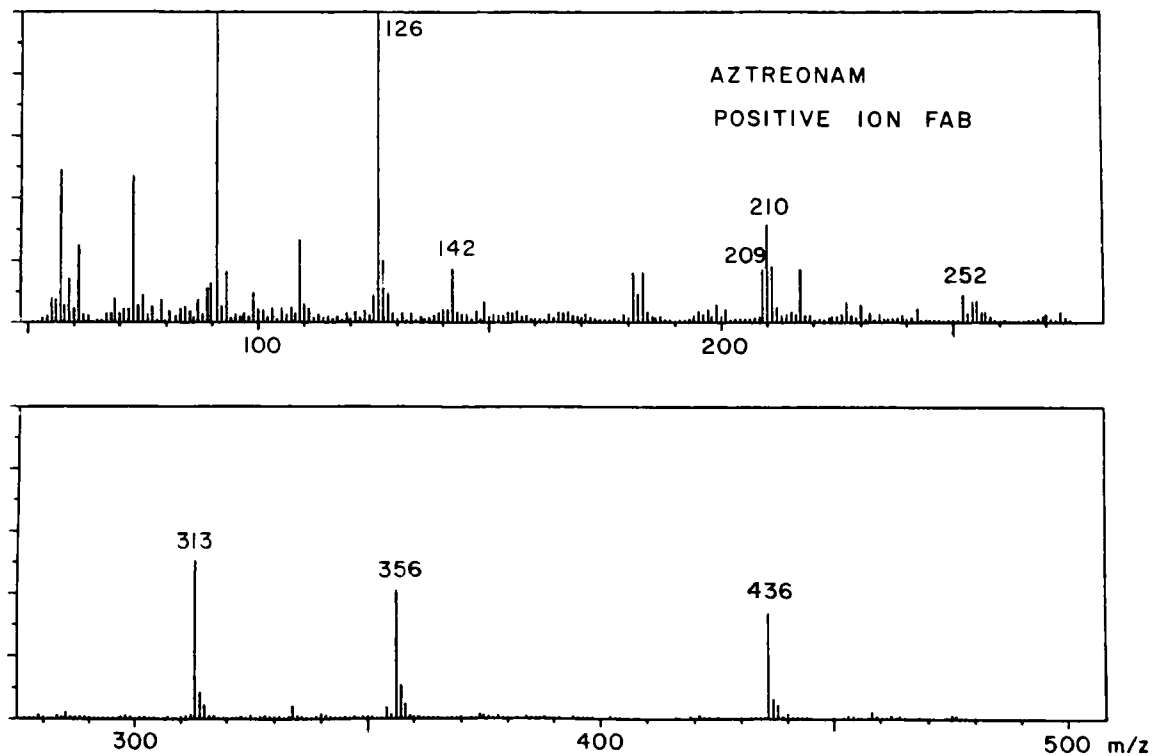


FIGURE 7. Positive Ion FAB-MS Spectrum of Aztreonam.  
Instrument: ZAB-IF-VG Analytical Ltd.

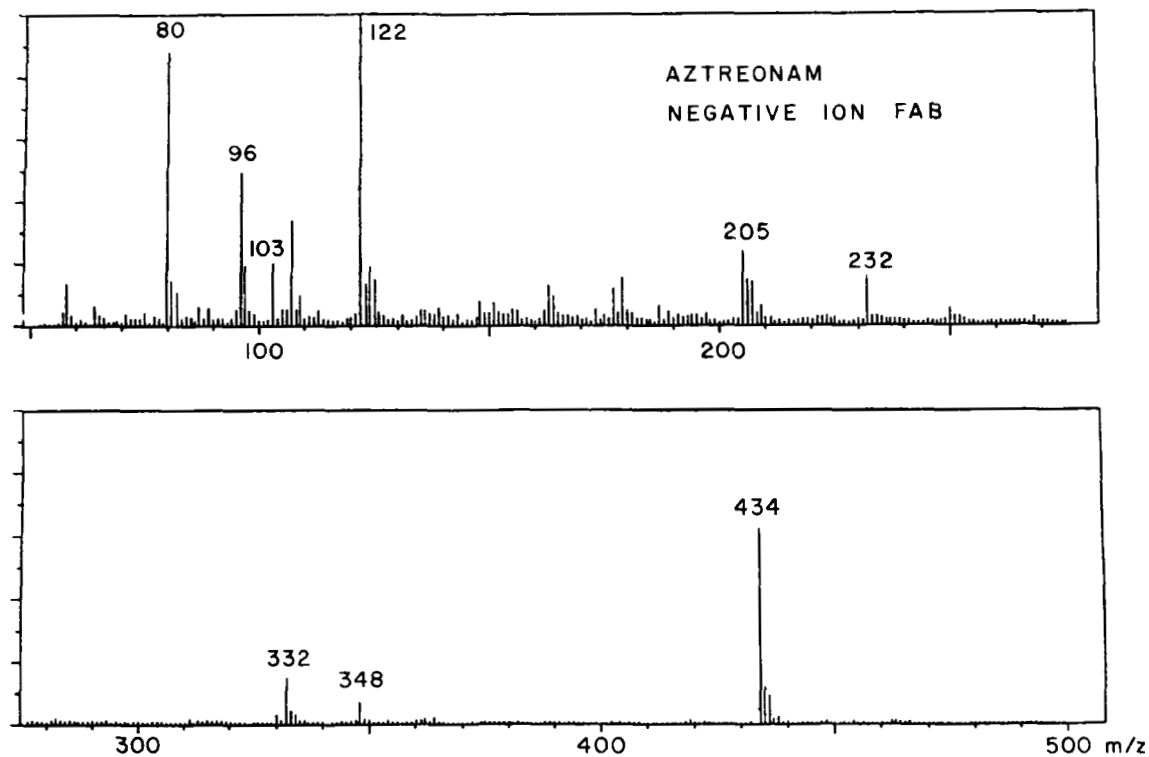


FIGURE 8. Negative Ion FAB-BS Spectrum of Aztreonam.  
Instrument: ZAB-IF-VG Analytical Ltd.



In the negative ion spectrum, the ions resulting from the direct N-O bond cleavage yields the  $[m/z\ 332]^-$  ion and its  $[m/z\ 103]^-$  ion complement. The principal high mass fragment ion in the positive ion mass spectrum results from the loss of sulfur trioxide from the  $MH^+$  ion. Cleavage of the monobactam ring of I gives rise to the  $313^+$  ion in the positive ion detection mode and its  $[m/z\ 122]^-$  complement in the negative ion detection mode.

The fragmentation patterns for the positive and negative mode<sup>13,14</sup> have been schematized in Figures 9 and 15. Thermospray mass spectra of aztreonam have been produced<sup>107</sup>, but due to extensive degradation, off-line HPLC, combined with FAB, has been found more useful<sup>16</sup>.

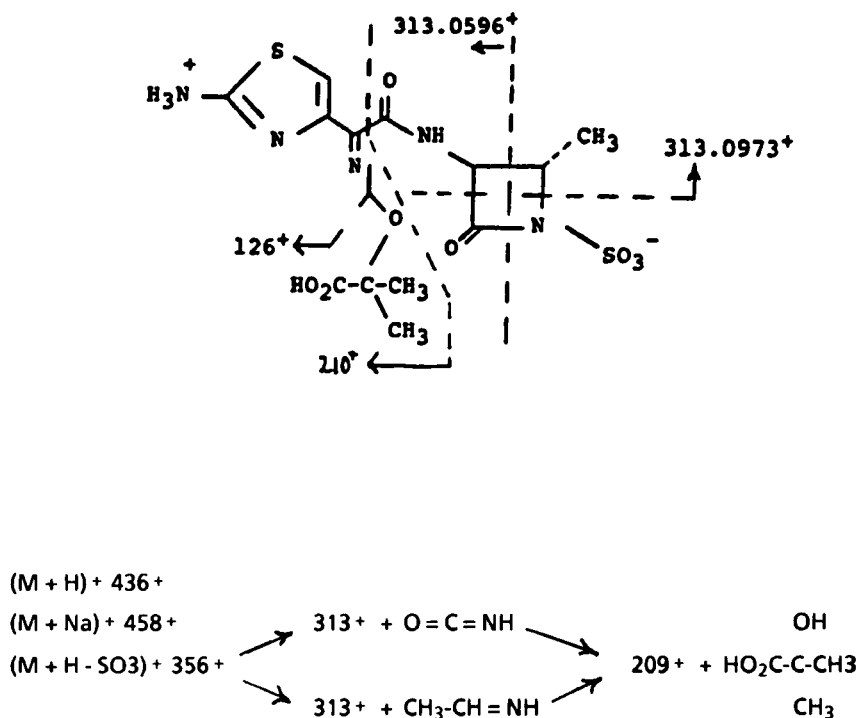
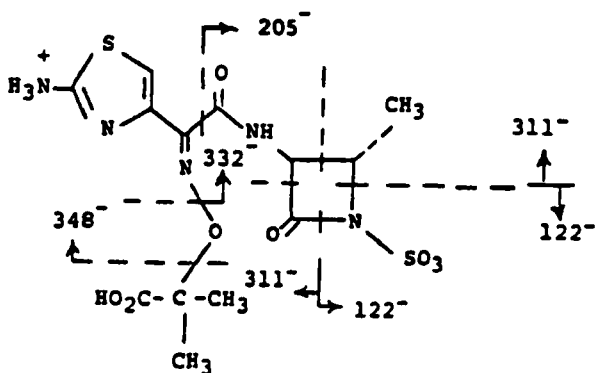


FIGURE 9. Positive Ion FAB Spectra of Aztreonam



(M-H)<sup>-</sup> 434<sup>-</sup>

(M-H-SO<sub>3</sub>)<sup>-</sup> 354<sup>-</sup> → 311<sup>-</sup> + } O=C=NH /or  
CH<sub>3</sub>-CH=NH

**FIGURE 10. Negative Ion FAB Spectra of Aztreonam**

### 3.4 Ultraviolet Spectra

The ultraviolet spectra of Research Standard Batch AZ028 in water (conc. 50.91 mg/100 x 4/100 (pH.4)) and in methanol (conc. 50.41 mg/100 x 6/100 ml) are presented in Figure 1117.

The absorptivities are as follows:

<u>Water</u>		<u>Methanol</u>	
max	E(1%; 1 cm)	max	E(1%; 1 cm)
233	248	235	244
258	237	264	278
285	222	280	251

The ultraviolet characteristics of aztreonam are pH dependent<sup>18</sup> (Figure 12).

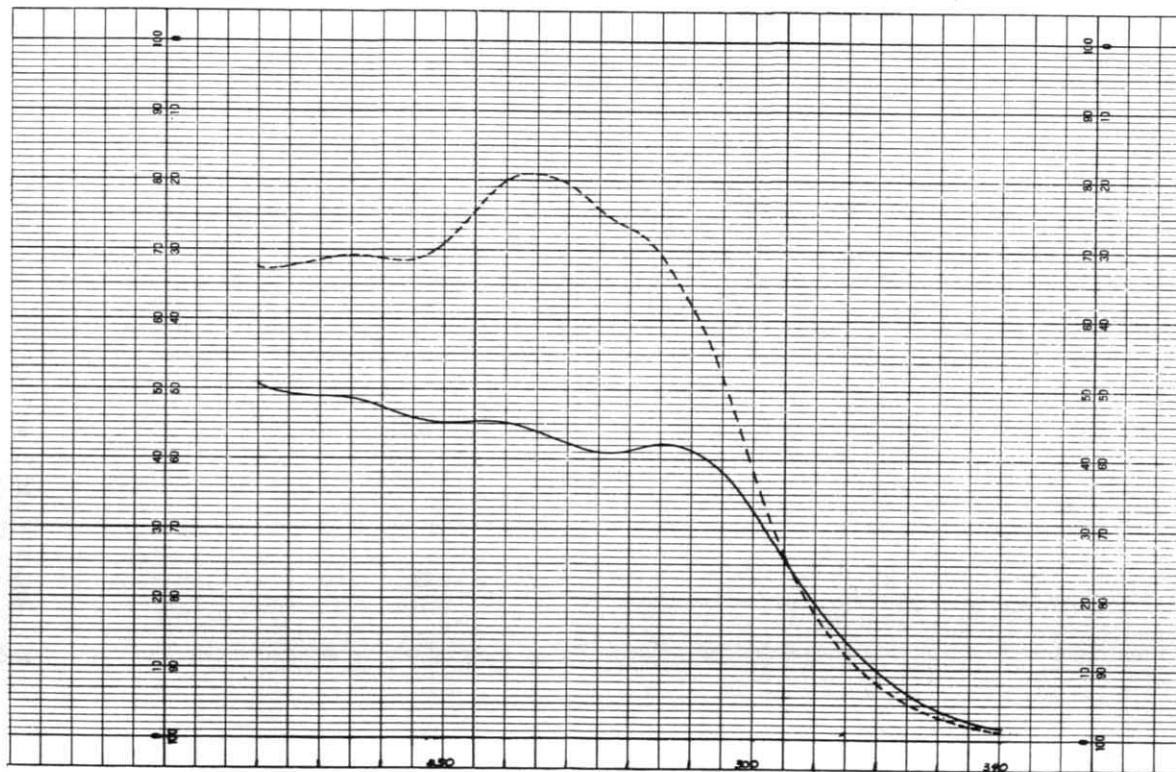


FIGURE 11. Ultraviolet Spectra of Aztreonam (Batch AZ028) in Water and Methanol.

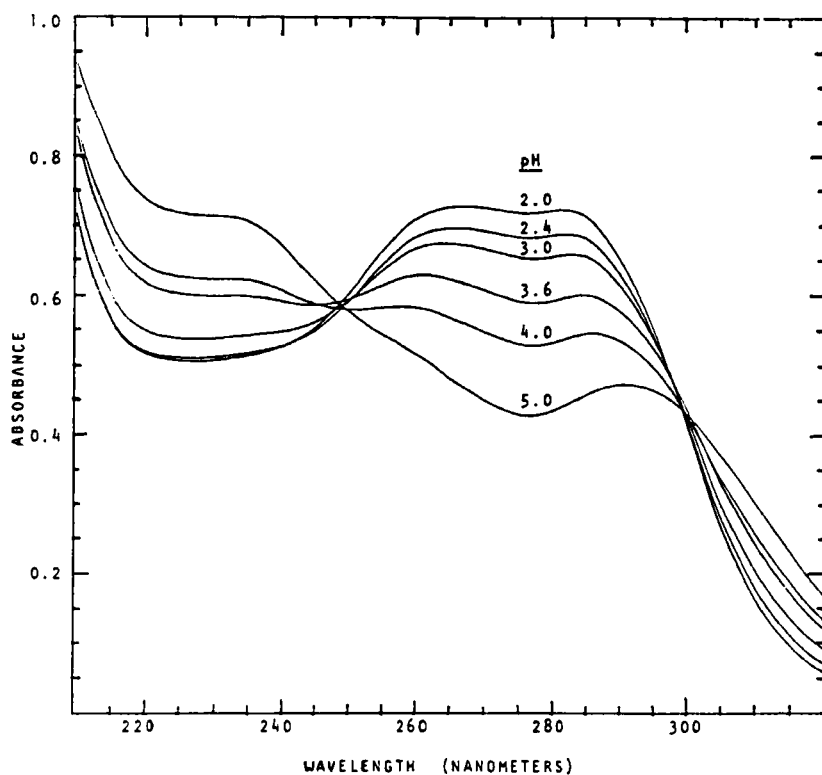


FIGURE 12. UV-Absorption Spectra of Aztreonam as a Function of pH.  
(25° C,  $5.7 \times 10^{-5}$  M, ionic strength 0.5 M)

### 3.5 Optical Rotation

Optical rotation of Research Standard Batch AZ028 in water (0.5%):  $[\alpha]_D -27.4^\circ$ .

### 3.6 Melting Range

Aztreonam melts with decomposition at  $\sim 227^\circ\text{C}$ .<sup>19</sup>  
 $\alpha$ -crystals:  $\sim 200^\circ\text{C}$ .  
 $\beta$ -crystals:  $\sim 240^\circ\text{C}$ .

### 3.7 Differential Scanning Calorimetry

The DSC curves of  $\alpha$  and  $\beta$ -forms of aztreonam are presented in Figure 13. The  $\beta$ -polymorph exhibits a sharp exotherm at  $240^\circ\text{C}$ .

All curves were obtained using the following parameters:<sup>20</sup>

Scan Rate	= $10^\circ\text{C}/\text{min}$ .
Range (sensitivity)	= $5\text{m cal/sec}$ .
Initial Temperature	= $310^\circ\text{K}$ ( $37^\circ\text{C}$ .)
Final Temperature	= $540^\circ\text{K}$ ( $267^\circ\text{C}$ .)

### 3.8 Thermal Gravimetric Analysis

TGA for two crystal forms of aztreonam gave the following loss of weight.

1. Alpha - 14.1% at  $200^\circ\text{C}$ .
2. Beta - 1.3% at  $200^\circ\text{C}$ .

Samples were heated at a rate of  $20^\circ\text{C}/\text{min}$ . to  $200^\circ\text{C}$ ., and their weight loss due to volatilization is recorded as percent<sup>20</sup>.

### 3.9 Ionization Constant, pK

The apparent  $\text{pK}_a$ 's of aztreonam at RT were determined by the method of Peck and Benet (*J. Pharm. Sci.* 67, 12 (1978)) which uses the X-intercepts in a plot of  $\log_{10} (S/S_0) - 1$  versus pH as the initial estimates of the apparent  $\text{pK}_a$ 's<sup>18</sup>.  $S_0$  is the lowest solubility measured. The  $\text{pK}_a$  values of the sulfonyl, amine and carboxyl groups are -0.7, 2.75 and 3.91, respectively. In the pH range of 0 to 10, aztreonam can exist as neutral zwitterion monoanion and dianion. Equilibria can be presented as follows<sup>19</sup>:



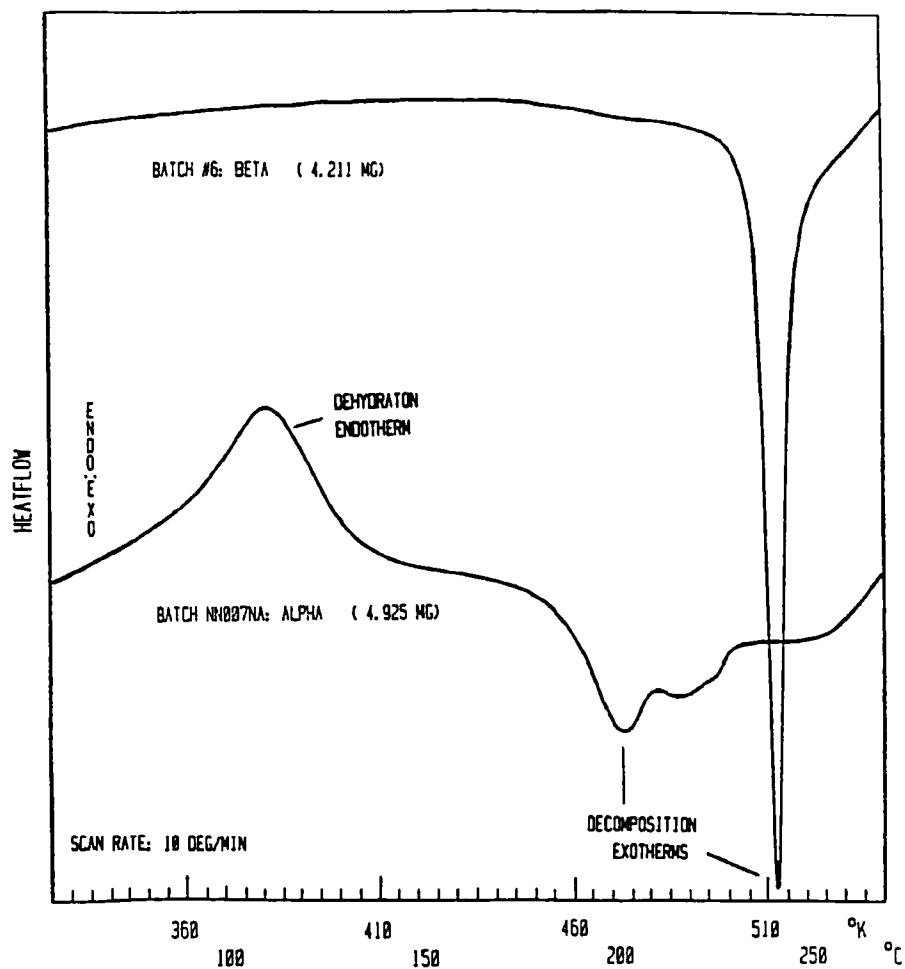


FIGURE 13. DSC Curves of Alpha and Beta Forms of Aztreonam.

These pK values are in good agreement with values obtained by potentiometric, spectrophotometric and kinetic methods<sup>18</sup>.

### 3.10 Solubility

In aqueous solution, aztreonam displays minimum solubility near its isoelectric pH of 2.25 (~10 mg/ml)<sup>21</sup>.

The pH-solubility profile shown in Figure 14 demonstrates that the solubility of the zwitterion of aztreonam is limiting at pH 1-3, but as ionization occurs, solubility increases dramatically at pH > 3. Solubilities of 40-50% w/v may be achieved at pH's as low as 4-5 and maintained even under refrigerator conditions.

The curve in Figure 14 at RT is described as follows:

$$S = S_o' [1 + 10 (pH - pK_{a2}') + 10 (2pH - pK_{a2}' - pK_{a3}') + 10 (pK_{a3}' - pH) ]$$

where S is the total solubility and  $S_o'$  is the apparent intrinsic solubility of the uncharged or zwitterionic form of aztreonam (see also 3.9).

Solubility of the  $\beta$ -form in organic solvents:

Methanol - 0.35% w/v<sup>19</sup>  
Ethanol - 0.02% w/v<sup>19</sup>

Solubility according to USP terminology:<sup>22</sup>

Methanol	- slightly soluble
Ethanol	- very slightly soluble
DMF	- soluble
DMSO	- soluble
Toluene	- insoluble
CHCl <sub>3</sub>	- insoluble
EtOAc	- insoluble

### 3.11 Crystal Properties, Polymorphism (see also Sections 3.1, 3.7 and 5.1)

Aztreonam has been observed in three distinct crystalline forms:  $\alpha$ ,  $\beta$  and  $\epsilon$ . All are pseudopolymorphs. The  $\alpha$ -form is obtained from aqueous solutions and is not very stable. It contains about 10-14% water (by K.F.). The crystals are fluffy rods and needles. The powder x-ray diffraction pattern is presented in Figure 15 and Table 3<sup>23</sup>. Small amounts (>11%) of  $\alpha$ -form in  $\beta$ -form can be detected semiquantitatively by x-ray powder diffraction<sup>24</sup>. For a DSC pattern, see Section 3.7.

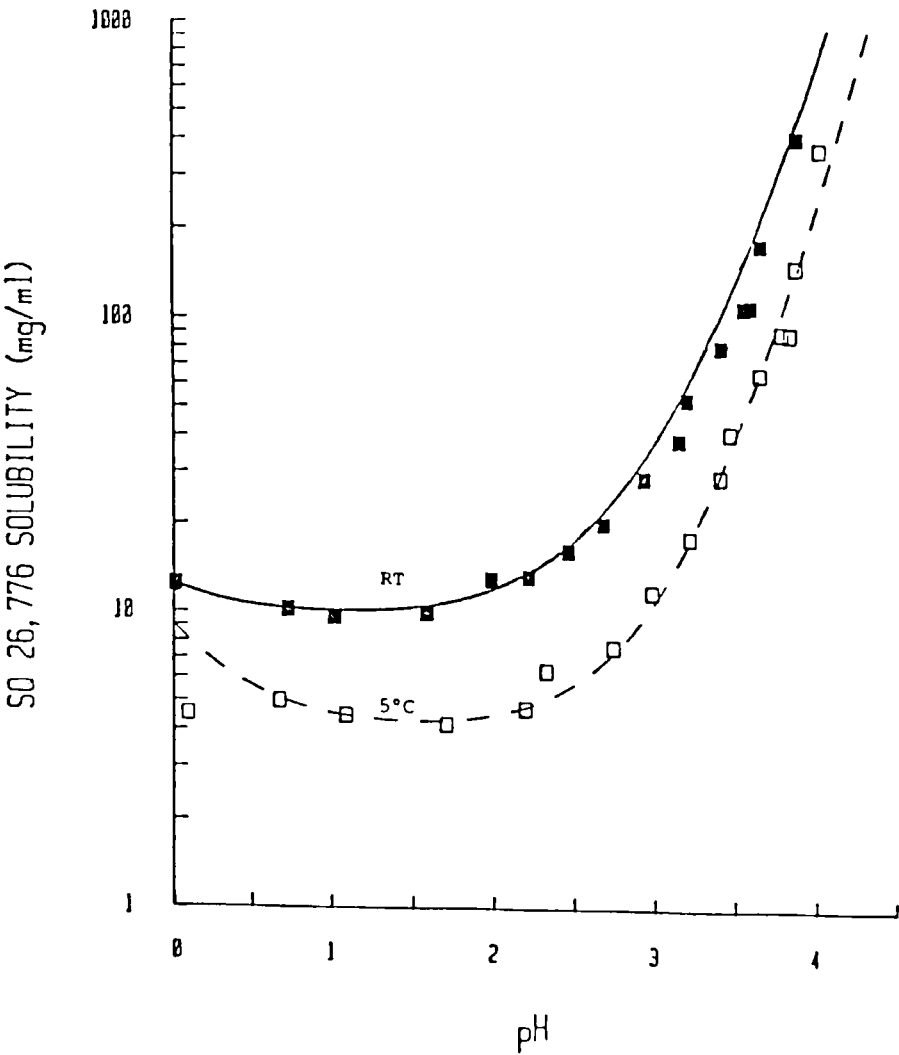


FIGURE 14. Aztreonam Solubility vs. pH at RT and 5° C., Ionic Strength = 0.5 (KCl).



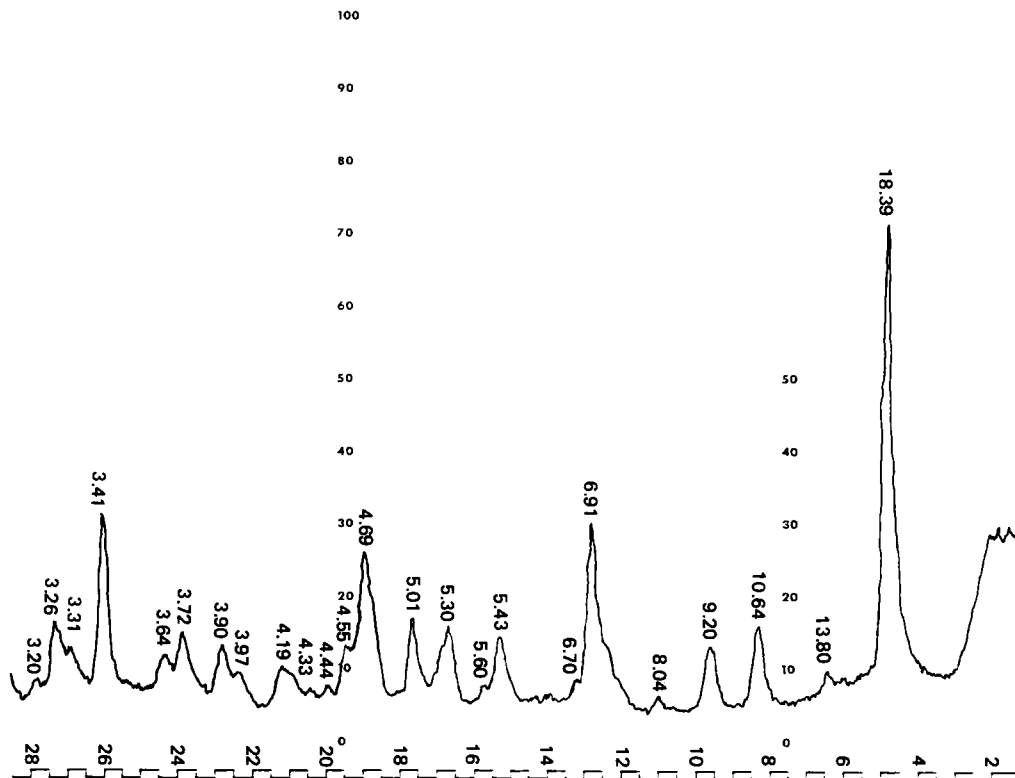


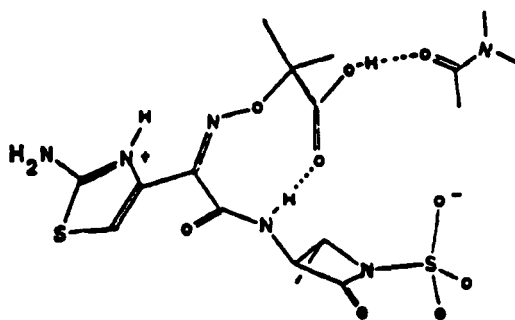
FIGURE 15. Powder X-Ray Diffraction Pattern of Aztreonam,  $\alpha$  - Form.

**TABLE 3****Powder X-Ray Diffraction Pattern of Aztreonam,  $\alpha$ -Form**

<b><u>Deg. 2<math>\theta</math></u></b>	<b><u>d (Å)</u></b>	<b><u>I (Peak Height)</u></b>	<b><u>I/I<sub>0</sub> (Relative Peak Height)</u></b>
4.8	18.39	63 (10)	1.00
6.5	13.80	3	0.05
8.3	10.64	11	0.17
9.6	9.20	8	0.13
11.0	8.04	2	0.03
12.8	6.91	25	0.40
13.2	6.70	1	0.02
15.3	5.43	9	0.14
15.7	5.60	1	0.02
16.7	5.30	11	0.17
17.7	5.01	12	0.19
18.9	4.69	21	0.33
19.5	4.55	8	0.13
20.0	4.44	2	0.03
20.4	4.33	2	0.03
21.2	4.19	5	0.08
22.4	3.97	4	0.06
22.8	3.90	8	0.13
23.9	3.72	10	0.16
24.4	3.64	7	0.11
26.1	3.41	26	0.41
26.9	3.31	8	0.13
27.3	3.26	11	0.17
3.20	3.20	4	0.06

The  $\beta$ -form, which is obtained from  $\alpha$ -material by recrystallization from ethanol, is very stable and contains about 1-2% ethanol. The crystals are dense aggregates and clusters. The powder x-ray diffraction pattern is presented in Figure 16 and Table 423. For a DSC pattern, see Section 3.7.

The  $\varepsilon$ -form is an orthorhombic pseudopolymorph, consisting of a 1:1 solvate of aztreonam with dimethylacetamide. It is relatively stable but will not normally be encountered, since dimethylacetamide is not used for recrystallization. A single crystal x-ray analysis of the  $\varepsilon$ -form has been made<sup>26</sup>. In this form, aztreonam is zwitterionic with a proton on the cyclic nitrogen atom of the aminothiazole ring. An intramolecular H-bond occurs between the amide proton and the carbonyl oxygen atom of the carboxylic acid. Each molecule of dimethylacetamide is the receptor of an intermolecular H-bond from the carboxyl proton of aztreonam.



Crystal properties for the dimethylacetamide (1:1) complex are as follows<sup>26</sup>:

$$a = 11.726(5) \text{ \AA}, \quad b = 22,139(8) \text{ \AA}, \quad c = 9.920(3) \text{ \AA} \\ \alpha = 90^\circ, \quad \beta = 90^\circ, \quad \gamma = 90^\circ, \quad v = 2575(3) \text{ \AA}^3$$

$d_{\text{obs}} = 1.32 \text{ g cm}^{-3}$ . Method or comments: Flotation in hexane/ $\text{CCl}_4$

$d_{\text{calc.}} = 1.35 \text{ g cm}^{-3}$  for  $Z = 4$  and formula of asym. unit:  $\text{C}_{17}\text{H}_{26}\text{N}_6\text{O}_9\text{S}_2$

Formula:  $\text{C}_{13}\text{H}_{17}\text{N}_5\text{O}_8\text{S}_2 \cdot \text{C}_4\text{H}_9\text{NO}$  Space Group:  $\text{P}2_12_12_1$

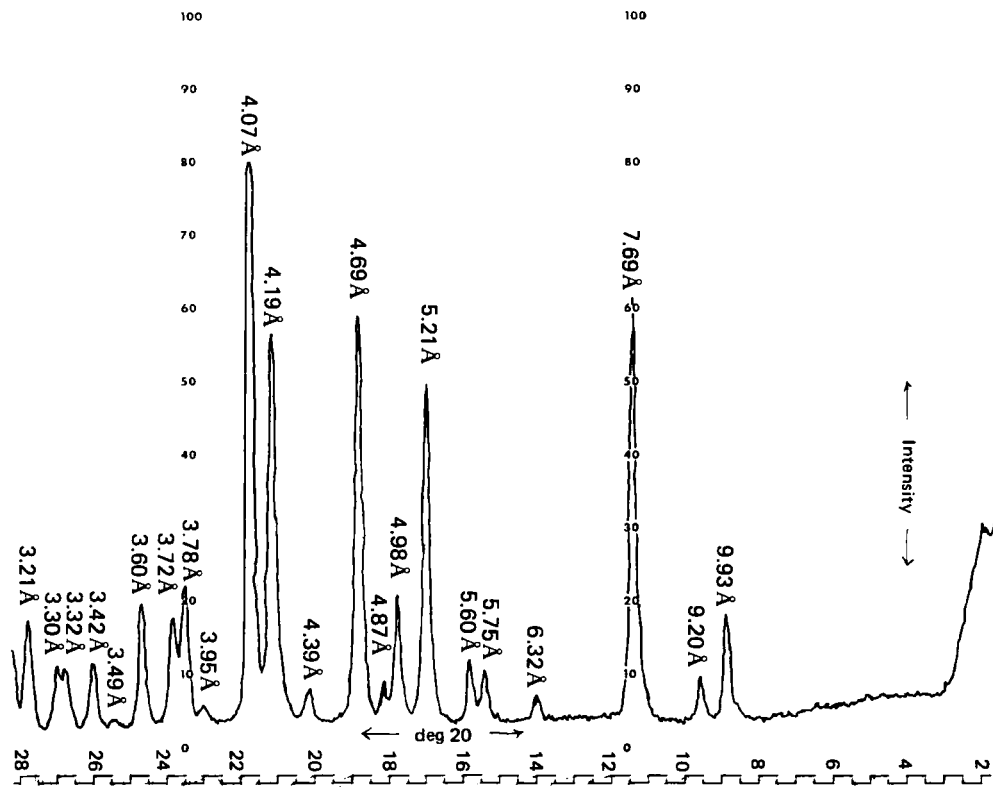


FIGURE 16. Powder X-Ray Diffraction Pattern of Aztreonam,  $\beta$ -Form, Batch AZ028.

**TABLE 4**  
**Powder X-Ray Diffraction Pattern of Aztreonam,  $\beta$ -Form**  
**BATCH #AZ028**

<u>Deg. 2<math>\theta</math></u>	<u>d (Å)</u>	<u>I</u> <u>(Peak Height)</u>	<u>I/I<sub>0</sub></u> <u>(Relative Peak Height)</u>
27.8	3.21	13	.17
27.0	3.30	7	.09
26.8	3.32	7	.09
26.0	3.42	8	.11
25.5	3.49	1	.01
24.7	3.60	16	.21
23.9	3.72	14	.18
23.5	3.78	18	.24
23.0	3.95	2	.03
21.8	4.07	76	1.00 I <sub>0</sub>
21.2	4.19	53	.70
20.2	4.39	4	.05
18.9	4.69	55	.72
18.2	4.87	9	.12
17.8	4.98	17	.22
16.0	5.21	46	.61
15.8	5.60	8	.11
15.4	5.75	6	.08
14.0	6.32	3	.04
11.5	7.69	58	.76
9.6	9.20	6	.08
8.9	9.93	14	.18

#### 4. Methods of Analysis

##### 4.1 Elemental Analysis

Calculated for  $C_{13}H_{17}N_5O_8S_2$

Found for Batch #AZ028  
(Res. Standard)

	<u>%</u>	<u>(After Drying)</u>
C	35.8	35.77
H	3.9	4.07
N	16.1	15.82
S	14.7	14.58

##### 4.2 Microbiological Assay

The basic microbiological agar diffusion assay method for aztreonam uses E. coli S.C. #12155 as organism, U.S.P. agar Medium #1, and phosphate buffer pH 6 (U.S.P. #6) as diluent<sup>27</sup>. U.S.P. Medium #2 has also been employed<sup>28</sup>.

The assay can be used for confirmation of chromatographic assays of bulk and formulation. It has found its greatest use in body fluid assays when high sensitivity (0.06 mcg/ml) is required <sup>27, 28</sup>.

##### 4.3 Iodometric Analysis

The well-known iodometric analysis for  $\beta$ -lactam was tried unsuccessfully for aztreonam<sup>29</sup>.

##### 4.4 Ultraviolet Analysis

Ultraviolet absorbance at 310 nm has been used to follow the dissolution of aztreonam capsules in 0.1M HCl<sup>30</sup>.

##### 4.5 Colorimetric Analysis

The alkaline hydroxylamine-ferric nitrate automated method for  $\beta$ -lactams has been adapted to aztreonam to assay powders and solutions. It was shown to be linear over a concentration of 377 to 1887 mg/ml<sup>31</sup>.

## 4.6 Chromatographic Analysis

### 4.61 Thin-Layer

Solvent systems to detect aztreonam are shown in Table 5. Aztreonam can be detected under short wave U.V. light.

### 4.62 High Performance Liquid

Several systems have been developed for aztreonam. They are based on:

1) Reversed phase columns ( $C_{18}$ ) with a mobile phase consisting of a mixture of low pH (mostly pH3) phosphate buffer containing tetrabutyl ammonium hydrogen sulfate (TBAHS) with acetonitrile in approximately 80:20 ratio<sup>27,35,54</sup>. A  $C_8$  column has also been used<sup>36</sup>, and so has been methanol instead of acetonitrile in the mobile phase<sup>37</sup>.

2) A reversed phase column ( $C_{18}$ ) with a mobile phase consisting of acetonitrile, ammonium acetate and tetrabutylammonium bromide (TBAB) at a ratio of 33:10:5 at a pH of 7<sup>38</sup>.

3) A normal phase (silica) column, using 0.1% orthophosphoric acid and 3% acetonitrile in water<sup>39,40</sup>. This system has also been used preparatively<sup>16</sup>.

Retention times in these systems vary from 1 to 10 minutes. Detection by U.V. absorption has been carried out at 210, 220, 254, 280 and 293 nm. Detection limits of 0.1  $\mu$ /ml have been achieved. The various systems have been used to determine stability of bulk drug and dosage form, as well as pharmacokinetics in biological fluids.

### 4.63 Electrophoretic

Three electrophoretic systems were used to determine aztreonam<sup>33</sup>:

**TABLE 5**  
**R<sub>f</sub> Values of SQ 26,776 after Thin-Layer Chromatography in Different Solvent Systems**

	<b><u>Plate</u></b>	<b><u>Solvent System</u></b>	<b><u>R<sub>f</sub> Value</u></b>	<b><u>Ref</u></b>
1	0.25mm Silica Gel GF (Analtech)	n-Propanol/acetic acid/water/ethyl acetate (70:2:35:60)	0.68	32
2	0.25mm Silica Gel GF (Analtech)	n-Propanol/acetic acid (9:1)	0.53	32
3	0.25mm Silica Gel GF (Analtech)	Chloroform/methanol/ammonia (50:50:5)	0.38 (very broad)	32
4	0.25mm Silica Gel GF (Analtech)	Chloroform/methanol/acetic acid/ethyl acetate (50:50:2:50)	0.26	32
5	0.25mm Silica Gel (Analtech)	Chloroform/methanol/methyl isobutyl ketone (1:1:1)	0.21 (very broad)	32
6	0.25mm SilicaGel GF (Analtech)	Ethyl acetate/methanol/acetic acid/methyl isobutyl ketone (50:50:2:50)	0.46	32
7	0.25mm Silica Gel GF (Analtech)	b-Butanol/ethyl acetate/water/acetic acid (1:1:1:1)	0.62	33
8	0.25mm Silica Gel GF (Analtech)	n-Butanol/acetic acid/water (3:1:1)	0.50	32
9	0.25mm Silica Gel 60 F254(Merck)	n-Propanol/acetic acid/water/ethyl acetate (70:2:35:60)	0.32	34
10	0.25mm Silica Gel 60 F254 (Merck)	n-Butanol/ethyl acetate/water/acetic acid (1:1:1:1)	0.50	33
11	0.25mm Silica Gel G (Analtech) impregnated with tetradecane	McIlvaine's buffer pH 6.5/acetone (200:3)	~0.5	34
12	0.25mm Silica Gel (Merck)	n-Propanol-ethyl acetate-pH 7.0 phosphate buffer (70:60:35)	~0.50	34
13	Analtech RPS	Water-acetonitrile-sodium perchlorate (995:5:0.7)	~0.80	33



<u>System</u>	<u>Support</u>	<u>Buffer/pH</u>	<u>Volts/cm</u>	<u>Minutes</u>
1	Cellulose (Eastman)	Pyridine acetate 0.05M, pH 4.0	20	60
2	Cellulose (Eastman)	2M Phosphate	10	45,81
3	Cellulose (Eastman)	Phosphate 0.05M, pH 6.9	20	60

## 5. Stability - Degradation

### 5.1 Solid Stability

The stability of  $\alpha$  and  $\beta$  crystalline forms has been studied (see 3.11 Crystal Properties).

#### 5.11 $\alpha$ -Form

The  $\alpha$ -form (crystallization from aqueous ethanol or methanol) is not very stable. A 1% loss at RT and an 80% loss at 80° C. after a one-week storage has been reported (Figure 17)<sup>41</sup>. An energy of activation of 25 kcal/mole, assuming a first-order model and 20 kcal/mole, assuming a zero-model was calculated. Both energies of activation were in the range indicative of hydrolysis reactions.

#### 5.12 $\beta$ -Form

The  $\beta$ -form (recrystallized from anhydrous ethanol) is stable. After a 12-month storage at -20°, +5°, +33°, +40°, +22°, 80% RH and 40° C./75% RH batches still pass specifications at all temperatures and humidity stations<sup>42</sup>. Even under the most rigorous storage conditions (40° C./75% RH), the samples were found to have undergone only a slight increase (<2% respectively by TLC) in impurities and a small drop (3.0 to 3.51% by HPLC) in potency.

### 5.2 Solution Stability

In aqueous solution, the most important source of instability of aztreonam over the whole pH range is hydrolysis of the beta-lactam ring<sup>19</sup>.

In weakly acid solutions (pH 2 to 5), hydrolysis is preceded by isomerization of the side chain as shown in Figure 18 (conversion of Z (syn) to E (anti) isomer)<sup>16,19</sup>. Care has to be exercised that E-isomer formation does not occur as an artifact

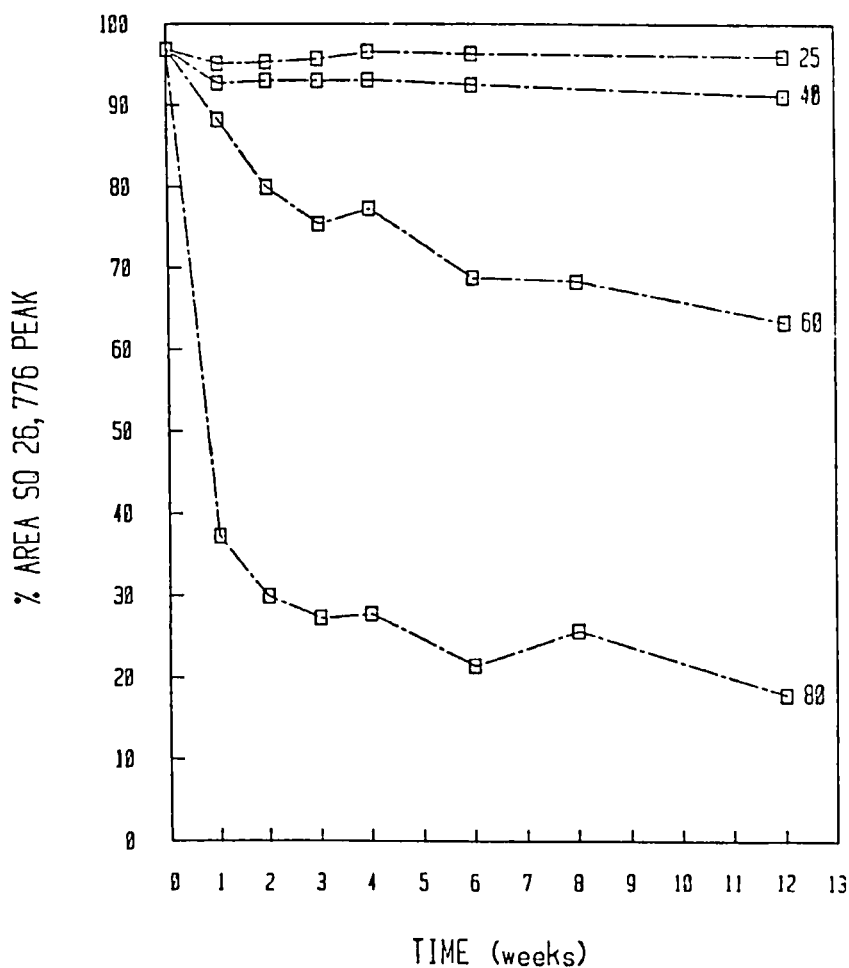


FIGURE 17. Aztreonam Alpha Form:  
Solid-State Stability: Temperature Profile

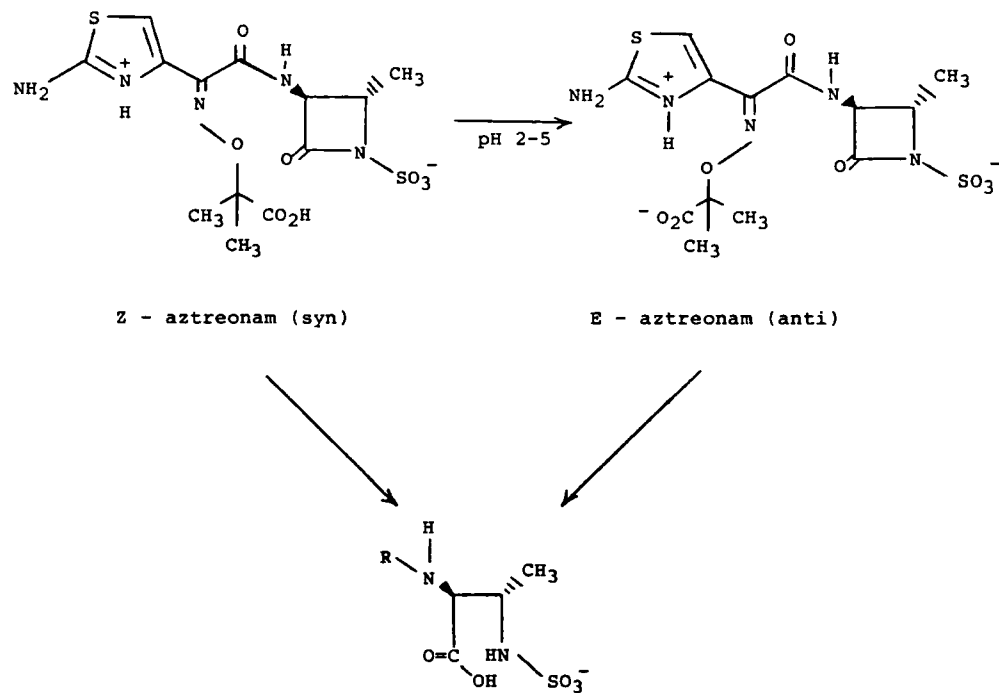
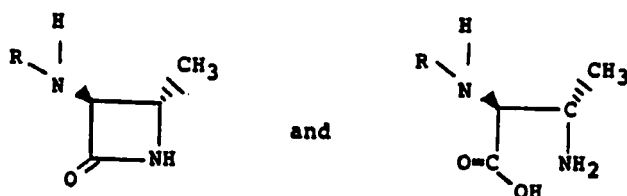


FIGURE 18. Degradation of Aztreonam in Aqueous Solution

during chromatographic separation<sup>43</sup>. The pH-rate profile for the hydrolysis of aztreonam at 35° C. and constant ionic strength ( $\mu = 0.5M$  KCl) is presented in Figure 19<sup>19</sup>. It shows that aztreonam is most stable in the pH 5-7 range. The Arrhenius plot for aztreonam degradation is presented in Figure 20<sup>19</sup>.

It has also been shown<sup>41</sup> that in aqueous, buffer-free solution, aztreonam is about 5 times more stable in the pH 4-7 range than most penicillins and cephalosporins, including ampicillin and cephadrine. At pH 5-7, aztreonam shows 10% degradation in 300-500 hours. Phosphate, tris, borate and carbonate buffers accelerate degradation.

Other degradation products that have been observed are the desulfonated products<sup>44</sup>:



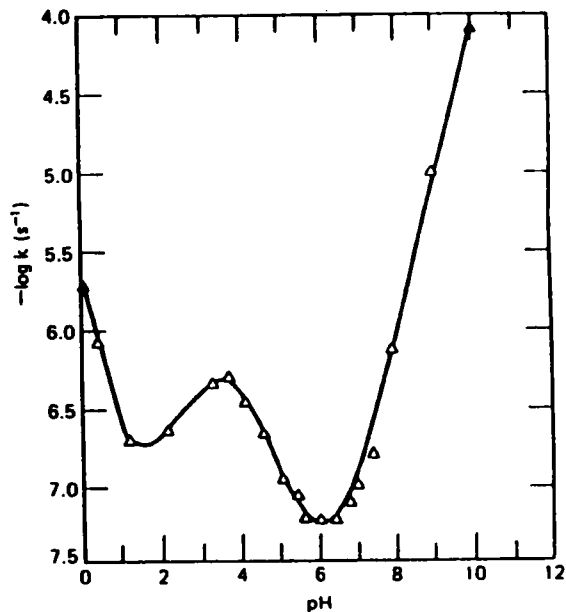
Degradation of aztreonam to dimers and trimers in aqueous solution has also been described<sup>45</sup>.

### 5.3 Light Stability

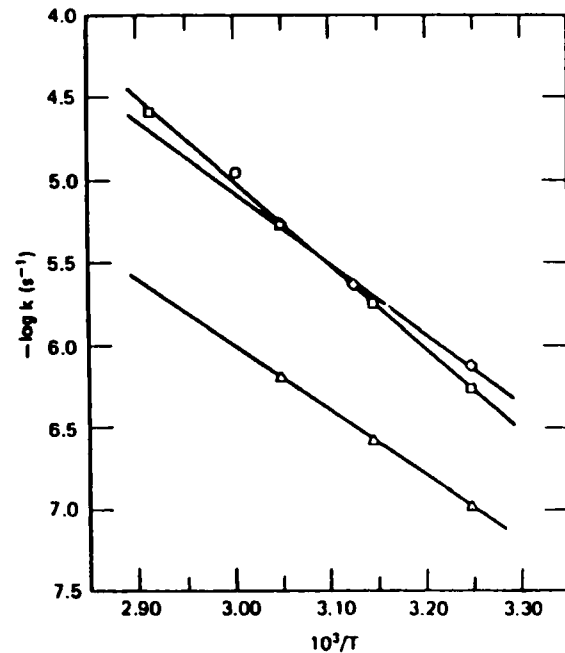
The  $\beta$ -crystals exposed one week to 400 and 900 foot-candles of fluorescent illumination and 33-35° C. temperature yellowed and showed a 6% conversion to the 'E' isomer. Samples stored in the dark at 33° C. or exposed to room light and temperature for one week showed no yellowing or chromatographic evidence of significant 'Z' to 'E' isomerization<sup>19</sup>.

### 5.4 Pink Discoloration

After long standing at a pH of <5, a pink discoloration of aztreonam has been observed. Solids exposed to moisture may also exhibit this phenomenon<sup>19</sup>.



**FIGURE 19:** AZTREONAM. pH Rate Profile for Degradation of Aztreonam at 35°C. an  $\mu = 0.5\text{M}$ . The rate constants have been extrapolated to zero buffer concentration.



**FIGURE 20:** AZTREONAM. Arrhenius Plots for Aztreonam Degradation.  
Key: □ pH 3.66; △ pH 5.40; ◇ pH 7.92

### 5.5 Stability in Biological Fluids

Aztreonam is stable in human serum and urine for at least 10 months when kept at  $-78^{\circ}\text{C}$ .<sup>46</sup>

However, aztreonam is unstable in human serum even at  $-20^{\circ}\text{C}$ . where a loss of 5% was observed after 7 days. However, immediate dilution (1/20) in pH 6.0 phosphate buffer greatly increased stability at  $-20^{\circ}\text{C}$ . to a loss of  $<10\%$  after a 2 month storage<sup>47</sup>.

Aztreonam is more stable in urine. It loses about 2.5%/24 hours at  $25^{\circ}\text{C}$ ., 1.5%/24 hours at  $5^{\circ}\text{C}$ . and only 0.5-15%/2 weeks at  $-20^{\circ}\text{C}$ . It, therefore, should be stored at  $-20^{\circ}\text{C}$ . and assayed within 4-6 weeks<sup>47</sup>.

Aztreonam was found to be stable in hemodialysis and peritoneal dialysis fluids for at least four days at room temperature to  $-20^{\circ}\text{C}$ .<sup>43</sup> There was no loss of activity in waste dialysate buffered to pH 6 after storage of  $-20^{\circ}\text{C}$ . for seven weeks. However an activity loss of  $\sim 9\%$  occurred in the unbuffered dialysate after two weeks' storage<sup>46</sup>.

## 6. Drug Metabolism, Pharmacokinetics

Metabolism and pharmacokinetics of aztreonam have been studied with  $^{14}\text{C}$  labeled aztreonam in the rat, dog and monkey<sup>48</sup> as well as in human volunteers<sup>48,49</sup>. Distribution in male and female rats<sup>50</sup>, in rat tissue<sup>51</sup> and fetuses and milk of rats<sup>52</sup> has also been described.

In human volunteers, after intravenous administration, the pharmacokinetic properties of aztreonam in serum were described by an open, linear, two-compartment model. After intramuscular administration of aztreonam to human volunteers, the serum concentration vs. time data was described by a biexponential equation which represented a one-compartment model with first-order absorption and elimination. Bioavailability after an intramuscular dose was 100%. After either intravenous or intramuscular administration, aztreonam was eliminated primarily by urinary excretion of unchanged aztreonam (about 66% of dose); only 1% of the dose was found as unchanged aztreonam in the feces, presumably as a result of biliary secretion. The values determined for the average elimination half-life of aztreonam were 1.6 and 1.7h, respectively, after intravenous and intramuscular administration. Aztreonam did not undergo extensive metabolism; the most prominent biotransformation product of aztreonam was the derivative resulting from the hydrolytic opening of the beta-lactam ring. Excretion of this compound in urine and feces accounted for 7 and 3% of the

administered dose, respectively. It was eliminated at a considerably slower rate than was aztreonam<sup>53</sup>. Other metabolites, accounting for 4 to 5% of the radioactivity in urine, have not yet been identified<sup>53</sup>.

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I also would like to thank Marie Bruno for competent and patient secretarial assistance.

# CYCLOBENZAPRINE HYDROCHLORIDE

MEREDITH L. COTTON AND G. R. BRIAN DOWN

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## 1. INTRODUCTION

### 1.1 History

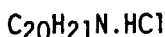
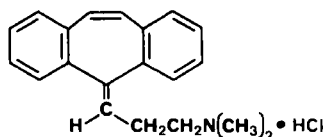
Cyclobenzaprine was synthesized by and the original patents assigned to Hoffmann-LaRoche & Co. (1, 2) in the late 1950's. Merck & Co. has patents or cross-licencing agreements in the United States, Canada, Chile, Peru and the Philippines. Originally the compound was tested for possible psychotherapeutic use but proved to be of less interest than its tricyclic analog, amitriptyline (3). Later, the hydrochloride salt of cyclobenzaprine (MK-130) was developed as a novel, centrally-acting, skeletal muscle relaxant by Merck Sharp & Dohme Research Laboratories (4-10).

It effectively and specifically reduced both  $\alpha$ - and  $\gamma$ - motorneurone efferent activity and a predominant supraspinal site of drug action was indicated (11). Cyclobenzaprine hydrochloride has been recommended as an adjunct to rest and physiotherapy for the relief of muscle spasm associated with acute, painful musculoskeletal conditions. At therapeutic doses, the compound does not affect CNS function. As a result, it depresses motorneurone hyperactivity without significant ataxia and is ineffective in muscle spasm caused by CNS disease (12, 13).

### 1.2 Name, Formula, Molecular Weight

The generic name (USAN) is cyclobenzaprine hydrochloride. The nomenclature is 1-propanamine, 3-(5H-dibenzo [a, d] cyclohepten-5-ylidene)-N, N-dimethyl, hydrochloride. The CAS registry number is 6202-23-9. Other names include N,N-dimethyl-5H-dibenzo [a, d] cyclohepten- $\Delta$  5, $\gamma$ -propylamine hydrochloride; 5-(3-dimethylaminopropylidene)dibenzo[a, e] cycloheptatriene hydrochloride; 1-(3-dimethylaminopropylidene)2,3:6,7-dibenzo-4-suberene:hydrochloride; 'LISSERIL'; 'FLEXERIL' and 'FLEXIBAN' (14, 15).

The structural formula of cyclobenzaprine hydrochloride is:



The chlorine content is 11.37% (w/w)

Its molecular weight is 311.85.

### 1.3 Appearance

Cyclobenzaprine hydrochloride is a white, practically odorless crystalline compound melting at 216-218°C.

## 2. PHYSICAL PROPERTIES

### 2.1 Infrared Spectrum

The infrared spectrum of cyclobenzaprine hydrochloride is shown in Figure 1. This spectrum was obtained using a Digilab Model 15C FT-IR spectrometer. The sample was prepared as a KBr pellet (1/200 mg) and the spectrum obtained from 128 scans (16). The characteristic bands of the spectrum with assignments are listed in Table I (17, 18).

TABLE I

Infrared Spectral Assignments for  
Cyclobenzaprine Hydrochloride

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignment</u>
3000-3100	C-H aromatic stretch
2900-2960	aliphatic C-H stretch
2100-2500 (broad singlet)	hydrochloride salt stretch characteristic of tertiary amines
1400-1500	N-CH <sub>3</sub> deformation
760-780	C-H aromatic out-of- plane deformation

### 2.2 Magnetic Resonance Spectra

#### 2.2.1 Proton Spectrum

A M/10 solution of cyclobenzaprine hydrochloride in deuterated chloroform or deuterium oxide was used to obtain a proton magnetic resonance spectrum (Figures 2, 3) employing a Bruker AM 300 spectrometer using tetramethylsilane as an internal chemical shift reference. The general characteristics of each spectrum with assignments are summarized in Table II (19, 20).

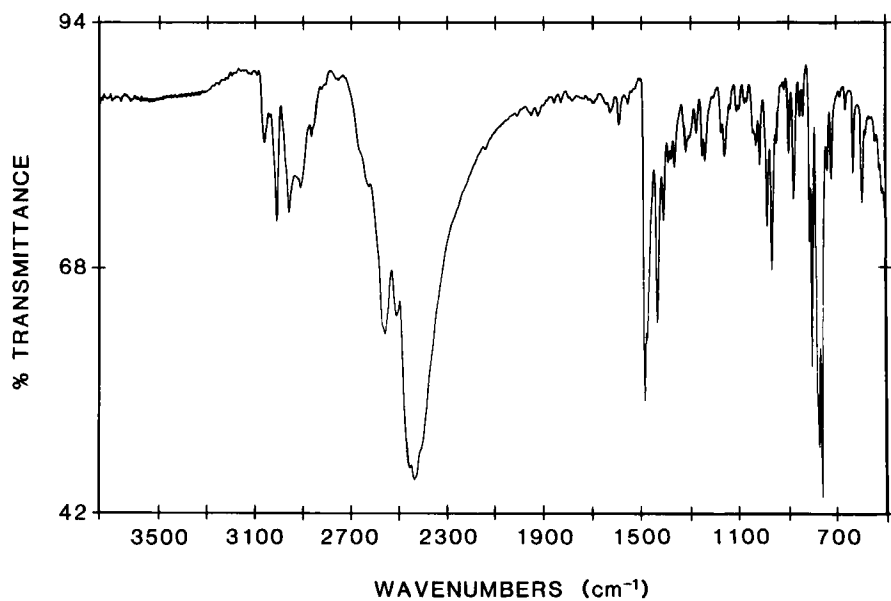


Figure 1. Infrared Absorption Spectrum of Cyclobenzaprine Hydrochloride.

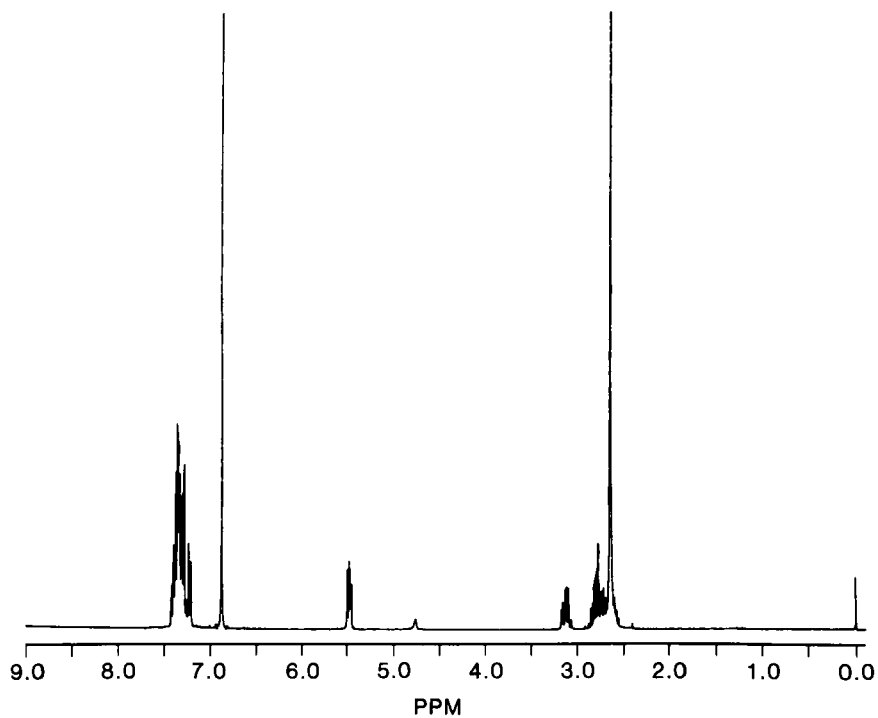


Figure 2. The Proton Magnetic Resonance Spectrum of Cyclobenzaprine Hydrochloride in CDCl<sub>3</sub>.

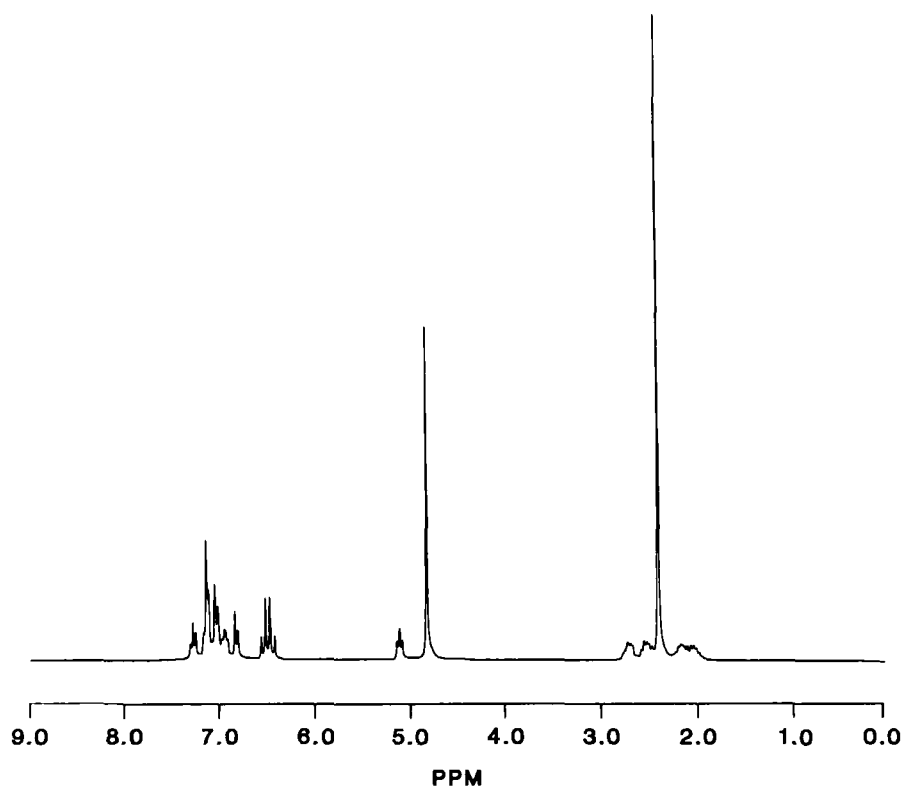
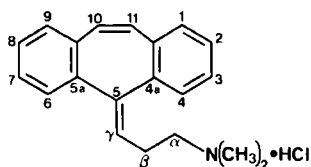


Figure 3. The Proton Magnetic Resonance Spectrum of Cyclobenzaprine Hydrochloride in  $D_2O$ .

TABLE II

Proton Magnetic Resonance Assignments  
for Cyclobenzaprine Hydrochloride



<u>Chemical Shift in CDCl<sub>3</sub>,<sup>δ</sup></u> <u>(ppm)</u>	<u>Assignment</u>
7.45 – 7.15 (m)	H(1-4), H(6-9)
6.86 (s)	H(10), H(11)
5.45 (dd)	H(γ)
3.10	H(α)
2.85 – 2.45	H(α'), H(β), H(β')
2.61 (s)	N(Me) <sub>2</sub>
<u>Chemical Shift in D<sub>2</sub>O,<sup>δ</sup></u> <u>(ppm)</u>	<u>Assignment</u>
7.01 (d)	H(1)
7.26 (dd)	H(2)
7.11 (m)	H(3)
7.01 (d)	H(4)
7.11 (m)	H(6), H(7)
6.93 (m)	H(8)
6.81 (d)	H(9)
6.43 (d)	H(10)
6.53 (d)	H(11)
2.59 (m)	H(α), H(α')
2.08 (m)	H(β), H(β')
5.09 (dd)	H(γ)
2.37 (s)	N(Me) <sub>2</sub>

### 2.2.2 Carbon-13 Spectrum

The carbon-13 spectra (Figures 4, 5) were obtained using the same conditions as those indicated for the proton spectra at a frequency of 75 MHz. The spectral assignments are listed in Table III (19, 20).



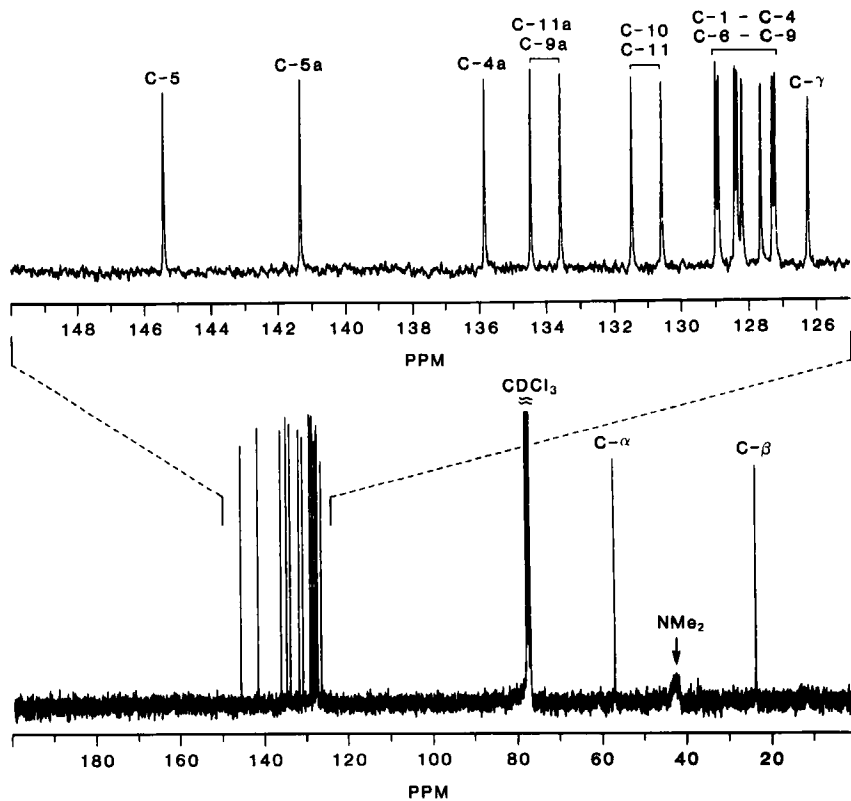


Figure 4. The Carbon-13 Magnetic Resonance Spectrum of Cyclobenzaprine Hydrochloride in CDCl<sub>3</sub> with Scale Expansion in the Region of 150-125 PPM.

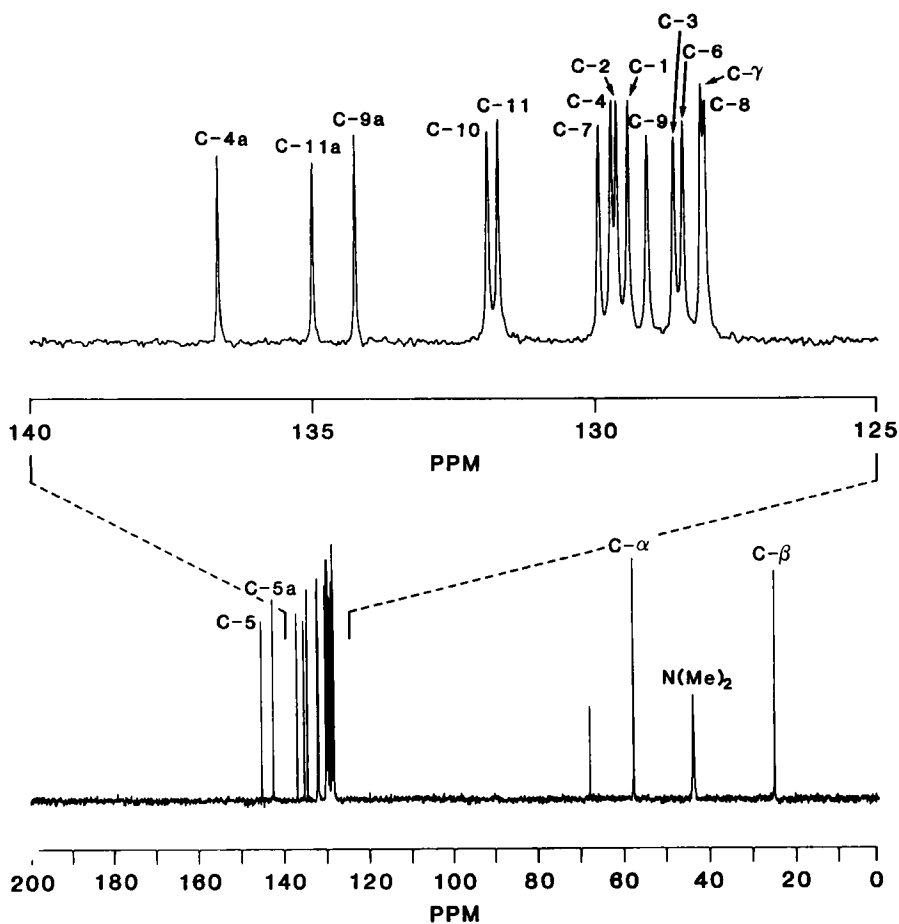


Figure 5. The Carbon-13 Magnetic Resonance Spectrum of Cyclobenzaprine Hydrochloride in  $\text{D}_2\text{O}$  with Scale Expansion in the Region of 140-125 PPM.

TABLE IIICarbon-13 Magnetic Resonance Assignments  
for Cyclobenzaprine Hydrochloride

<u>Chemical Shift in CDCl<sub>3</sub>, δ (ppm)</u>	<u>Assignment</u>
145.36	C-5
141.26	C-5a
135.75	C-4a
134.39	C-11a
133.49	C-9a
131.40	C-10
130.52	C-11
128.91	C-1 - C-4 C-6 - C-9
128.83	
128.34	
128.27	
128.15	
127.58	
127.25	
127.18	
126.21	C-γ
56.71	C-α
42.38	N(Me) <sub>2</sub>
23.41	C-β
<u>Chemical Shift in D<sub>2</sub>O, δ (ppm)</u>	<u>Assignment</u>
129.38	C-1
129.59	C-2
128.59	C-3
129.68	C-4
136.65	C-4a
144.99	C-5
142.33	C-5a
128.43	C-6
129.90	C-7
128.05	C-8
129.06	C-9
134.24	C-9a
131.86	C-10
131.67	C-11
134.99	C-11a
57.21	C-α
24.39	C-β
128.11	C-γ
43.09	N (Me) <sub>2</sub>

In the  $\text{CDCl}_3$  solvent, further evidence for assignment of the kinetically broadened peak to the methyl nitrogen groups was obtained. Treating the solution with an excess of hydrogen chloride produced a sharp pair of peaks at 42.5 and 43 ppm. The addition of hydrogen chloride effectively retarded deprotonation of the nitrogen, a necessary precedent to inversion at the nitrogen. Under these conditions with the non-planar fused ring framework and the two methyl groups not equivalent, a well defined doublet would be expected to occur. Assignment of the C- $\gamma$  and C-10, C-11 carbons was based on off-resonance proton decoupling experiments.

### 2.3 Ultraviolet Spectrum

One of the original ultraviolet absorption spectra of cyclobenzaprine hydrochloride was obtained using a Beckman DB-G grating spectrophotometer (Figure 6). The compound was dissolved in methanol (0.59 mg/100 ml) and run with methanol as the reference. The spectrum obtained was characterized by two maxima at 225 nm and 290 nm with absorptivity values,  $A$  (1%, 1cm) values, of 1240 and 343, respectively (18). Other spectra obtained were in good agreement with these values (17, 21). The broad absorption with a maximum at 290 nm was assigned to the endocyclic double bond and the shoulder at 240 nm was assigned to the exocyclic double bond (22).

### 2.4 Mass Spectrum

The mass spectrum of cyclobenzaprine hydrochloride was obtained using a Finnigan MAT 212 mass spectrometer operating in the electron impact mode with an ionizing potential of 90 eV (23). The primary features of the mass spectral data are presented in the customary normalized bar graph shown in Figure 7. These results are in agreement with spectral data published elsewhere (24-26). A proposed fragmentation pattern is shown in Figure 8.

The mass at  $m/e$  275 is the molecular ion. Fragmentation at the methylene-methylene bond produces the mass at  $m/e$  58, the most abundant ion, and the mass at  $m/e$  217 reported earlier as a primary fragment (24). At conditions under which the current data were obtained, the major fragment having an  $m/e$  of 215 is best considered as a stable fragment resulting from the dehydrogenation and cyclation of the remaining side-chain. This fragment can generate either the mass at  $m/e$  189 by loss of ethylene or the mass at  $m/e$  202, the cyclooctatetraene analog.

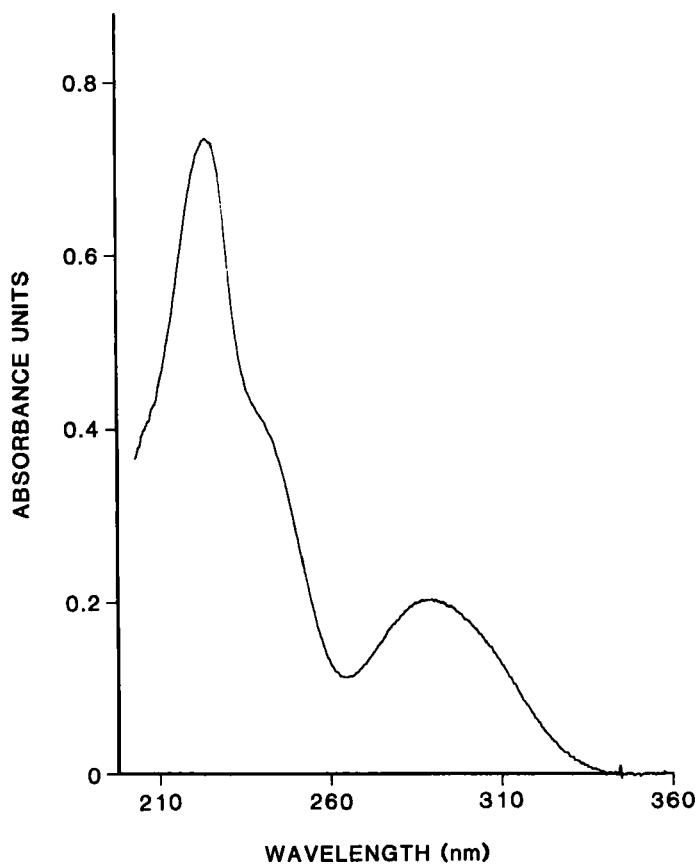


Figure 6. The Ultraviolet Absorption Spectrum of Cyclobenzaprine Hydrochloride.

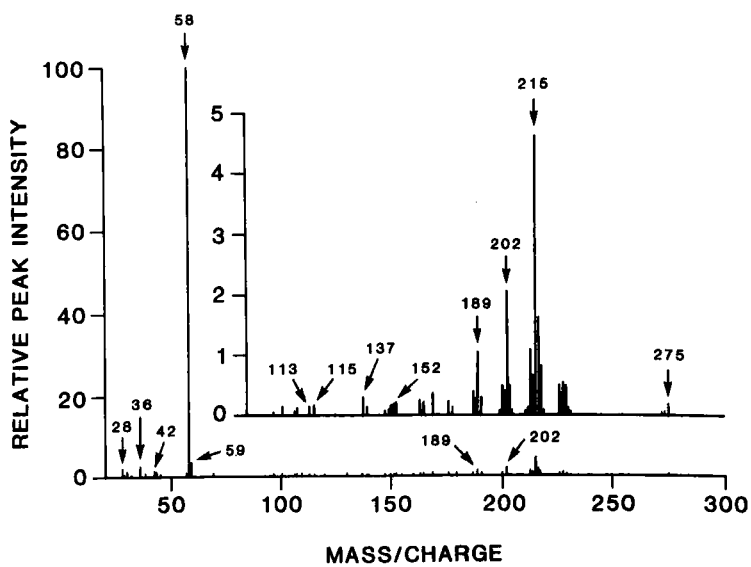


Figure 7. The Electron-Impact Mass Spectrum of Cyclobenzaprine Hydrochloride.

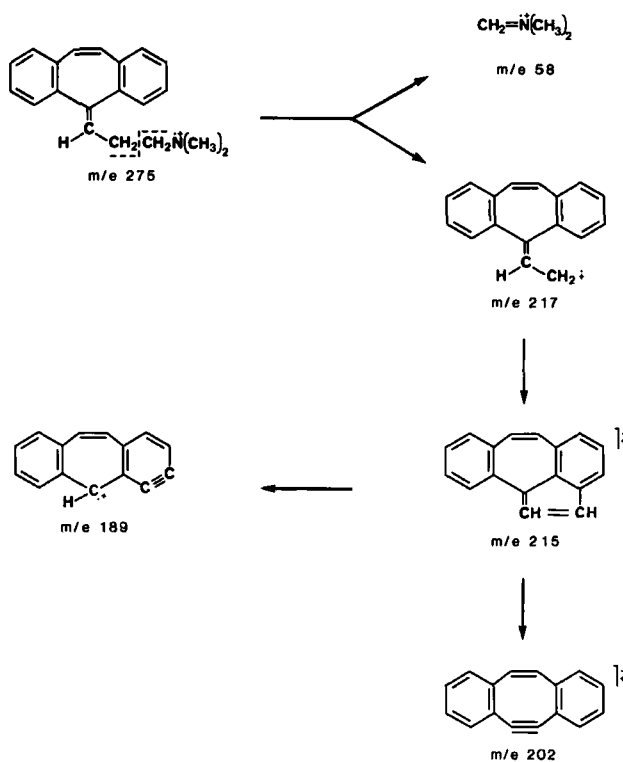


Figure 8. A Proposed Fragmentation Pattern to Explain the Mass Spectrum of Cyclobenzaprine Hydrochloride.

### 2.5 Thermal Behavior

Cyclobenzaprine hydrochloride has been reported to melt at 216–218°C (14). Using a Perkin–Elmer DSC–4 differential scanning calorimeter, a thermal curve obtained at a scan rate of 10 degrees/minute had a single endothermic response with an onset temperature of 217°C (Figure 9). Decomposition occurred following the melt. Results from 5 samples analyzed by DSC at a scan rate of 1 degree/minute in standard aluminum pans (6–10 mg/sample) indicated an average heat of fusion of 9.10 kcal/mole with a RSD of 1.1% (27). Average onset and maximum temperatures obtained were 217.0°C and 217.6°C, respectively.

### 2.6 Optical Behavior

Since the compound has no assymetric centers there is no optical activity.

### 2.7 Solubility

The compound as the hydrochloride salt is freely soluble in water and the free base may be precipitated by rendering the aqueous solution alkaline. The solubilities in a number of organic solvents are indicated in Table IV (18, 21).

TABLE IV

Solubilities of Cyclobenzaprine Hydrochloride

<u>Solvent</u>	<u>Solubility</u>
water	freely soluble
methanol	freely soluble
ethanol	freely soluble
isopropanol	sparingly soluble
chloroform	limited solubility
methylene chloride	limited solubility
hydrocarbons	insoluble

### 2.8 Crystal Properties

The compound is a white crystalline material when observed by optical microscopy. To date, only a single crystal form has been observed and characterized. No polymorphism has been observed by X-ray diffraction, thermal analysis, infrared spectroscopy or solubility analysis. No solvates have been observed to date. The X-ray powder diffraction data obtained using a diffractometer with copper K $\alpha$  radiation (1.54056 Å to 1.54435 Å) are shown in Table V and Figure 10 (28).



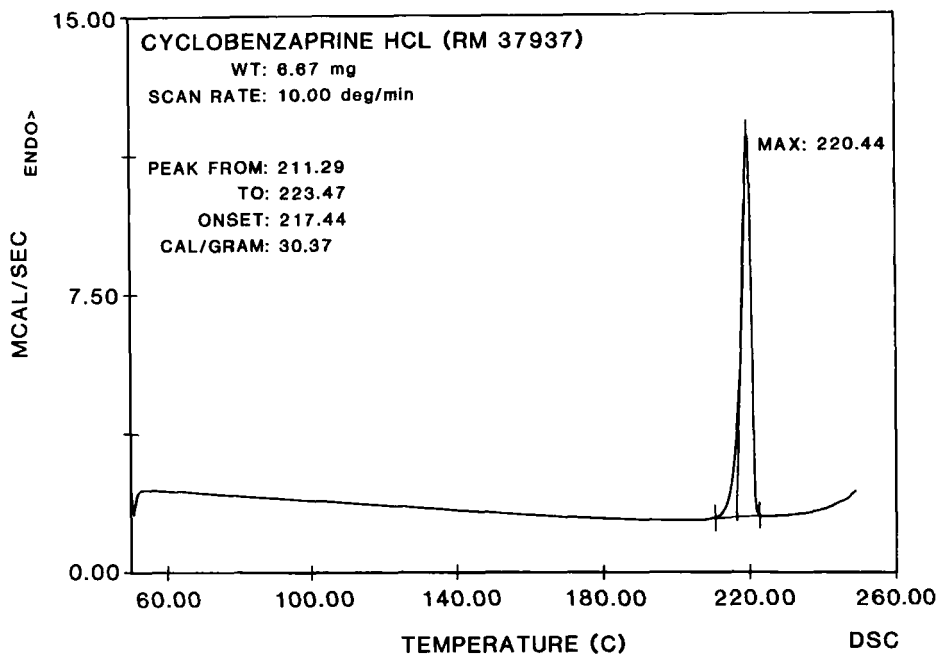


Figure 9. A DSC Thermal Curve of Cyclobenzaprine Hydrochloride.

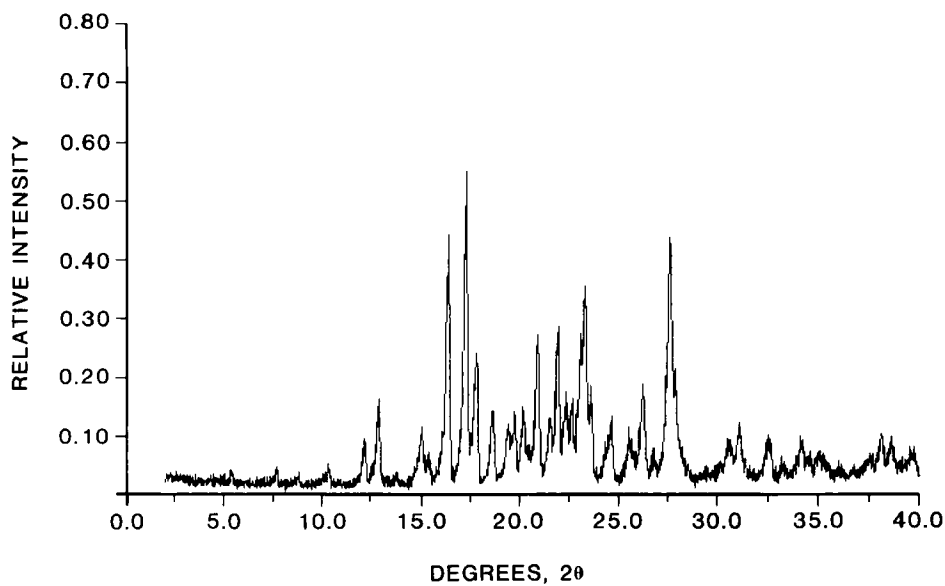


Figure 10. The X-ray Powder Diffraction Pattern for Cyclobenzaprine Hydrochloride.

TABLE VX-ray Diffraction Data for the Single Crystal Form  
of Cyclobenzaprine Hydrochloride

<u>2<math>\theta</math>(deg)</u>	<u>d(Å)</u>	<u>I/I<sub>0</sub>(%)</u>	<u>2<math>\theta</math>(deg)</u>	<u>d(Å)</u>	<u>I/I<sub>0</sub>(%)</u>
12.21	7.24	14.93	22.74	3.91	24.10
12.97	6.82	28.28	23.13	3.84	40.50
15.09	5.87	18.65	23.38	3.80	61.84
16.48	5.38	77.76	23.70	3.75	21.92
17.38	5.10	100.0	24.69	3.60	18.26
17.90	4.95	43.44	25.62	3.47	11.93
18.76	4.73	21.92	26.30	3.39	31.26
19.48	4.55	17.11	27.66	3.22	83.47
19.83	4.47	23.21	30.60	2.92	11.31
20.28	4.38	23.65	31.12	2.87	15.28
21.05	4.22	44.04	34.16	2.62	10.71
21.62	4.11	19.84	38.16	2.35	12.25
22.06	4.03	49.00	38.68	2.33	10.71
22.43	3.96	25.00			

2.9 Hygroscopicity

Cyclobenzaprine hydrochloride is slightly hygroscopic. It equilibrates at ambient conditions adsorbing 0.5% moisture. This moisture may be removed by desiccating over phosphorus pentoxide with reduced pressure or heating to 60–100°C. To ensure that adsorption of water is not significant, it is recommended that relative humidities above 60% be avoided.

2.10 Acid Dissociation Constant

The pK<sub>a</sub> for the ionization at the amine group of cyclobenzaprine hydrochloride has been determined as 8.47 at 25°C (17).

3. SYNTHESIS

The preparation of cyclobenzaprine hydrochloride is carried out by reacting the Grignard reagent, prepared from 3-dimethylaminopropyl chloride (29), with dibenzo[a, e]cycloheptatriene-5-one to form the intermediate tertiary carbinol. This is dehydrated in the presence of hydrogen chloride to yield the final product. The synthesis, as shown in Figure 11, has been described in detail elsewhere (29, 30).

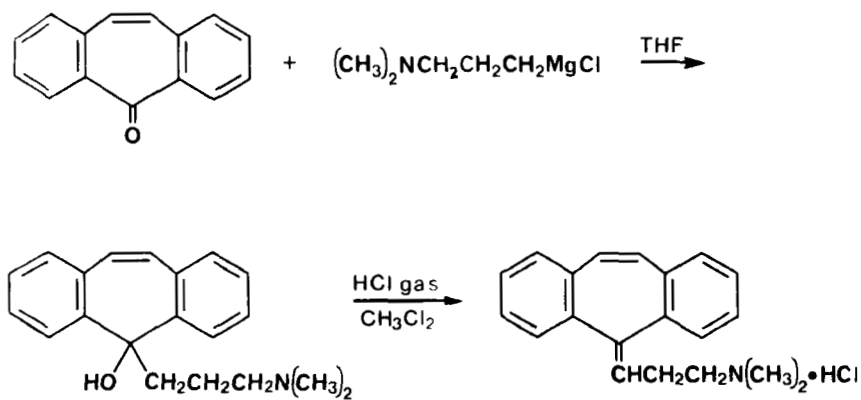


Figure 11. The Synthesis of Cyclobenzaprine Hydrochloride.

#### 4. STABILITY AND DEGRADATION

##### 4.1 Potential Routes of Degradation

Cyclobenzaprine hydrochloride degrades through oxidation of either (1) the endocyclic double bond (2) the exocyclic double bond to form an epoxide or (3) the oxidation of the nitrogen group to form the corresponding N-oxide. These inherently unstable, initial oxidation products undergo further transformation to more polar compounds and subsequent cleavage of the aliphatic side-chain to form dibenzocycloheptatrienone and anthraquinone as major oxidation products. The final result of degradation is a large number of different oxidation products, each present at trace concentrations. Only anthraquinone and dibenzocycloheptatrienone are present as preponderant degradation products. The proposed primary degradation pathways in acidic aqueous solutions are shown in Figure 12 (18, 31). A combination of these three basic reaction mechanisms produces a variety of intermediate products. This proposed scheme is similar to that for amitriptyline hydrochloride in aqueous solution (32-35). It has been reported that, despite saturation at the endocyclic double bond in the chemical structure of amitriptyline, anthraquinone is the primary product of potassium permanganate oxidation and not dibenzosuberone (33). The compound, dibenzocycloheptatriene, which is the analog of dibenzosuberone for cyclobenzaprine, is converted stoichiometrically to anthraquinone in aqueous solutions of mineral acids or in the presence of common organic oxidizing agents (18, 36).

##### 4.2 Solid-State Stability

There is no evidence of significant solid-state degradation in the bulk chemical or in the presence of common excipients under ambient or moderate accelerated stability conditions. Solid cyclobenzaprine hydrochloride stored at ambient conditions was stable for several years. No degradation products were observed in tablet formulations analyzed by TLC after 4 years at room temperature (36). Uncoated tablets after ten years at ambient conditions were analyzed by a stability-indicating HPLC assay method (31) (for details see section 6.3.3, Table VIII, item 3) and a wet chemical method involving the partitioning of the degradation products from a dilute sulfuric acid solution into methylene chloride and analysis of these decomposition products spectrophotometrically (18). No loss of drug or the presence of degradation products were observed. Tablet formulations and granules

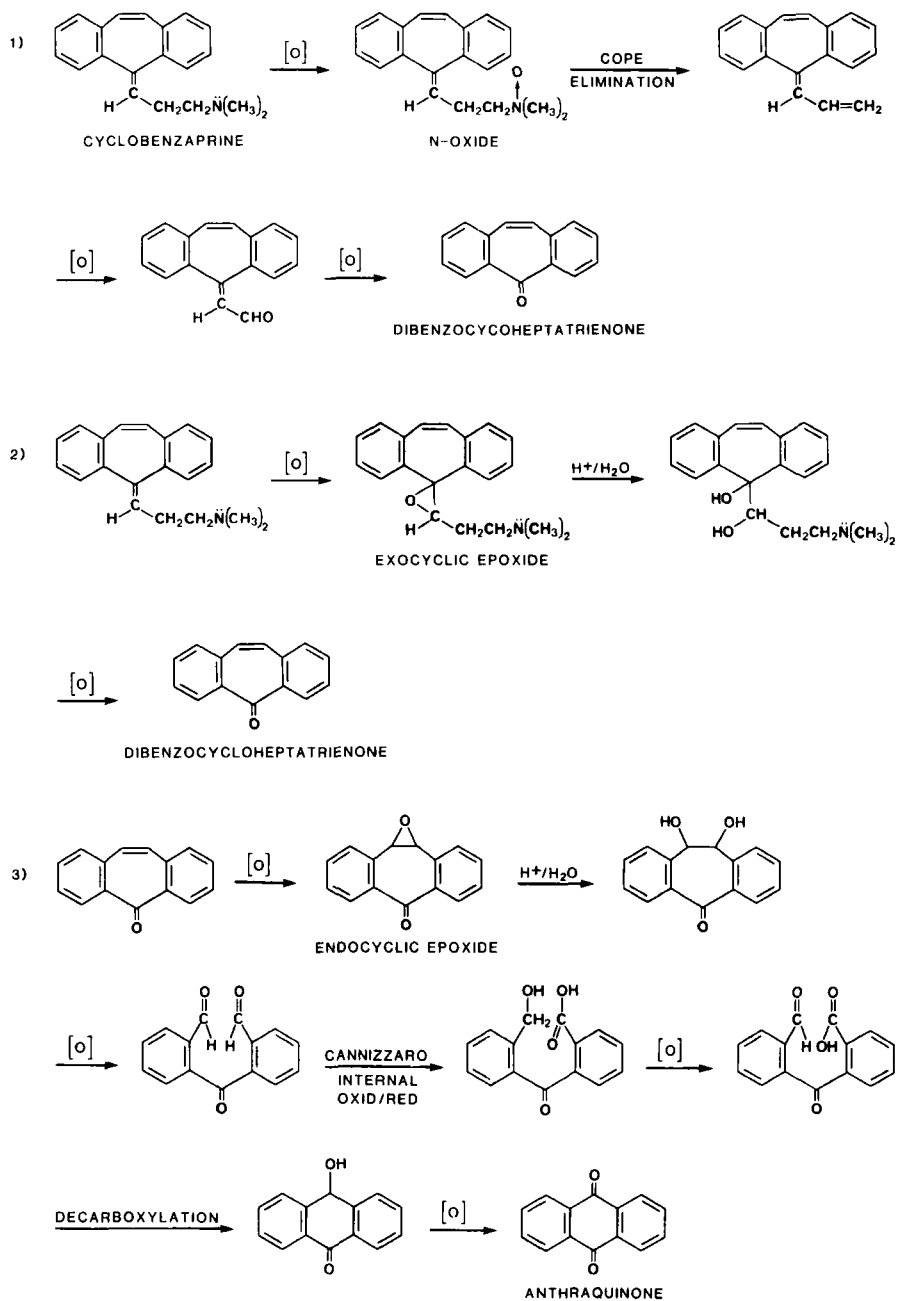


Figure 12. Proposed Degradative Pathways for Cyclobenzaprine Hydrochloride in Acidic Aqueous Solution.

stressed at 80°C for 2 and 4 weeks, when analyzed by HPLC, contained trace amounts of anthraquinone and dibenzocycloheptatrienone (less than 0.1%) (37).

There is evidence to indicate that when cyclobenzaprine hydrochloride degradation is observed in the solid state it occurs through an acid-catalyzed oxidation mechanism. With acetylsalicylic acid (ASA) present at about a cyclobenzaprine hydrochloride/ASA weight ratio of 1/50, compressed tablets, when analyzed by an HPLC stability assay method, showed 15–20% and 5–12% loss of intact cyclobenzaprine hydrochloride after 21 days at 80°C and 6 months at room temperature, respectively (38). Tablets containing the same excipients but excluding the ASA component showed no significant loss of drug under similar conditions. The primary degradation product was anthraquinone. However, trace amounts of a variety of other oxidation products accounted for at least 50% of the drug loss. This degradation could be eliminated by introducing butylated hydroxyanisole (BHA) into the compressed tablets providing further evidence for oxidative decomposition.

#### 4.3 Solution Stability

Degradation of cyclobenzaprine hydrochloride occurs under conditions of rather severe stress in aqueous solution. There is considerable evidence indicating that decomposition occurs primarily by an oxidative process under these conditions. Precipitates from solutions of the compound in N/10 hydrochloric acid stored at 95°C for about one year and in N/10 sulfuric acid stored for several weeks at room temperature were isolated and identified as anthraquinone (18). The exocyclic epoxide was obtained in about 0.4% yield, in addition to other oxidation products, by refluxing acetonitrile solutions of cyclobenzaprine hydrochloride containing hydrogen peroxide and ferrous nitrate (37).

Cyclobenzaprine hydrochloride, dissolved in 50% aqueous acetic acid and stirred for 3 days at 80°C with the headspace flushed with oxygen, was analyzed by HPLC and found to contain 56% of the original starting material as intact drug. A variety of oxidation products could be separated including anthraquinone and dibenzocycloheptatrienone. These latter two components accounted for about 50% of the total amount of drug lost (31). Two compounds

present in trace amounts were identified by UV and mass spectroscopy as substituted anthracenes probably a substituted aldehyde and a substituted carboxylic acid (31, 39).

## 5. PHARMACOKINETICS AND METABOLISM

### 5.1 Absorption and Elimination

Cyclobenzaprine hydrochloride is well absorbed in all species after oral administration (40-42). In the rat, it is widely distributed in all tissues and excreted primarily in the bile. In man, it is predominantly protein-bound in the plasma (41) and absorption is dose-dependent (42).

Elimination in the rat is primarily through the feces. Urinary excretion is predominant in the dog, the monkey and in man (41). Urinary excretion and plasma concentrations for oral and intravenous administration in man provided evidence for a route-dependent biotransformation (40, 42).

### 5.2 Metabolism

In man (42), as in other species (41, 24), the drug is extensively metabolized with less than one percent excreted intact in the urine. Comparison of areas under the curve from oral bioavailability studies indicate that the drug was extensively destroyed during first-pass metabolism (42). In man the major metabolites are a glucuronide of cyclobenzaprine and 10, 11-dihydroxynortriptyline (41). Major metabolites in the dog are the N-oxide, the 1, 2-dihydrodiol and 3-hydroxycyclobenzaprine, (41, 43, 24). Six metabolites were isolated from rat urine (44, 25). These were the 10, 11-epoxide, the N-oxide, the desmethyl derivative, hydroxycyclobenzaprine, demethyl-hydroxycyclobenzaprine and hydroxycyclobenzaprine N-oxide. Following the 'in vitro' metabolism (26) by rat liver microsomes (44, 45, 46), various metabolites isolated included the 10, 11-epoxide, the N-oxide, demethylcyclobenzaprine and 10, 11-epoxycyclobenzaprine N-oxide.

The 10, 11-epoxide metabolites have been shown to be nonmutagenic and noncytotoxic (47). These metabolites are considered to be stable detoxification products rather than intermediates in the drug biotransformation process (25, 48).



### 5.3 Interactions

Cyclobenzaprine hydrochloride may enhance the effect of nervous system depressants, such as ethanol, through the inhibition of alcohol dehydrogenase (49, 50). It may interact with monoamine oxidase inhibitors leading to hyperpyretic crises (51, 52, 53).

## 6. METHODS OF ANALYSIS

### 6.1 Identification Tests

There are three identification tests discussed in the USP (15). The infrared spectrum of a sample obtained in a mineral oil dispersion of previously dried material should show wavelengths and absorption bands corresponding to those of a similar preparation of USP reference standard. The ultraviolet absorption spectrum of a 1:65000 parts methanol solution should have maxima and minima at the same wavelengths as a similar solution of USP reference standard. The absorptivity on a dried basis at 290 nm for these two solutions should not differ by more than 3.0%. Finally, a 1:50 parts aqueous solution should respond positively to the USP chloride test.

### 6.2 Ultraviolet Spectrophotometric Analysis

Cyclobenzaprine hydrochloride is quantitatively determined in tablet formulations by extracting and dissolving the drug in N/10 aqueous sulfuric acid (15) or N/25 aqueous hydrochloric acid containing 60% methanol (18) and comparing the UV absorption with reference standard solutions at 290 nm.

### 6.3 Chromatographic Analysis

#### 6.3.1 Thin Layer Chromatography (TLC)

There are several reported TLC systems for cyclobenzaprine hydrochloride listed in Table VI. All these analytical systems used silica gel plates to chromatograph samples. Samples tested included bulk chemical, rodent diet mixtures and various biological samples. Detection systems employed include ultraviolet, fluorescence and radiographic techniques. The TLC methods listed often included quantitative determination by either isolating and analyzing material from developed plates or direct fluorescence emission densitometry (54).

TABLE VIA Summary of TLC Systems Used to Analyze  
Cyclobenzaprine Hydrochloride

<u>Developing Solvent</u>	<u>R<sub>f</sub></u>	<u>Reference</u>
1. acetone:ammonia (100:1)	0.56-0.60	41, 24, 55
2. benzene:dioxane:ammonia (60:35:5)	0.48-0.57	41, 24
3. chloroform:methanol:acetic acid (47.5:47.5:5)	0.55-0.61	41, 24
4. ammonia saturated chloroform: methanol (19:1)	0.76-0.88	41, 24
5. benzene:dioxane:ammonia (10:80:10)	0.86-0.88	41, 24
6. isopropanol:diethylamine (95:5)	0.51	26
7. cyclohexane:isopropanol:methanol: diethylamine (60:25:10:5)	0.72	44, 25, 46
8. cyclohexane:diethylamine:toluene: methanol (75:10:10:5)	0.50	44, 25, 46
9. chloroform:methanol:acetic acid (70:20:10)		54
10. ethylacetate:hexane:ammonia (90:10:12)	0.51	54
11. acetone:toluene:ammonia (75:25:1)		15
12. acetone:benzene:ammonia (100:30:1)		18
13. acetone:benzene:butanol:acetic acid:water (5:5:5:4:4)		18

### 6.3.2 Gas Chromatography (GLC)

There have been a number of GLC methods of analysis reported. These are listed in Table VII. Samples include extracts from plasma, urine and TLC plates. Generally, TMS derivatives were prepared prior to injection using standard techniques (56). Detection systems include flame ionization (FID), nitrogen selective detection (N) and mass spectroscopy (MS). The carrier gas used was generally nitrogen or helium.

TABLE VII

A Summary of GLC Systems Used to Analyze  
Cyclobenzaprine Hydrochloride

	<u>Liquid Phase</u>	<u>Support</u>	<u>Temp</u>	<u>Detector</u>	<u>Reference</u>
1.	3% OV-17	Gas-Chrom Q 100-120 mesh	240	N	57
2.	3% OV-17	Gas-Chrom Q 100-120 mesh	270	FID, MS	44, 25, 46
3.	3% OV-17	Chromosorb Q 100-120 mesh	250	FID, MS	26
4.	3% OV-17	Gas-Chrom Q 100-120 mesh	240	N	41
5.	1.5% OV-17	Gas-Chrom Q 80-100 mesh	240	FID	41
6.	1.5% OV-17	Gas-Chrom Q 80-100 mesh	220	FID	55
7.	1.5% OV-17	Gas-Chrom Q 80-100 mesh		FID	24
8.	1.5% OV-17	Gas-Chrom Q 80-100 mesh	218	FID	58
9.	1% OV-17	Gas-Chrom Q 80-100 mesh		FID	41
10.	1% OV-17	Supelcoport 80-100 mesh	200- 220	MS	41

11. 1% OV-17	Supelcoport 80-100 mesh	220- 240	MS	24
12. 3% OV-17	Supelcoport 80-100 mesh	250- 260	N, MS	59
13. SE-30	LKB capillary column type 2101-502	250	N	59
14. 3% QF-1	Chromosorb G (acid-washed silanized)	210- 240	FID	60

### 6.3.3 High Pressure Liquid Chromatography (HPLC)

Few HPLC methods have been reported for resolving cyclobenzaprine hydrochloride. This is in part due to drug development having been completed prior to HPLC being a generally accepted chromatographic method and in part due to its poor elution characteristics with conventional reversed-phase columns. The compound, in the absence of a suitable organic counter ion, eluted as an asymmetric peak presumably because of interaction with the silica packing. This peak asymmetry could be largely eliminated with octylamine in the acidic mobile phase. Three HPLC assay methods are listed in Table VIII.

TABLE VIII

#### A Summary of HPLC Systems Used to Analyze Cyclobenzaprine Hydrochloride

- Sample: Aqueous Methylcellulose Solutions  
 Column:  $\mu$ -bondapak C18, 10  $\mu$ m, (300 x 3.9) mm  
 Conditions: 40°C, 2 ml/min  
 Mobile Phase: 65% Acetonitrile in Formic Acid:Water (1:70)  
 Detection: 290 nm  
 Reference: 61
- Sample: Tablets and Injectables  
 Column: Ultrasphere, Octyl, 5  $\mu$ m, (250 x 4.6) mm  
 Conditions: 30°C, 2 ml/min  
 Mobile Phase: Water:Acetonitrile:Phosphoric Acid:Octylamine (1200:800:0.9:0.3)  
 Detection: 290 nm  
 Reference: 37

3. Sample: Bulk Chemical, Tablets  
Column: Ultrasphere, IP, 5  $\mu$ m, (250 x 4.6) mm  
Conditions: 40°C, 2 ml/min  
Mobile Phase: Water:Acetonitrile:Acetic Acid:Octylamine  
(800:200:6:1)  
Detection: 254 nm  
Reference: 31

#### 6.4 Nonaqueous Titration

A nonaqueous acid-base titration assay for purity of the bulk chemical is described in USP XXI (15). The sample is dissolved in glacial acetic acid and titrated with N/10 perchloric acid. The end-point is determined potentiometrically with a platinum ring-calomel electrode.

#### 6.5 Dye-Transfer Analysis

A dye-transfer technique, based on the work of French et al (62), has been used to analyze cyclobenzaprine hydrochloride in tablet formulations (18). The drug is extracted with aqueous solutions of N/10 hydrochloric acid and diluted to concentrations of 2-20  $\mu$ g/ml. An aliquot (5 ml) of this solution is neutralized with pH 7.0 phosphate buffer. An N/10 sodium hydroxide solution containing 0.04% w/v bromothymol blue (3 ml) is added to form an ion pair which is extracted into a toluene organic phase. The organic phase is analyzed spectrophotometrically at 405 nm and the concentration determined by comparing the absorbance with that of a reference standard prepared at a known concentration in a manner similar to that of the sample.

#### 6.6 Determination in Biological Fluids

Biological fluids have been analyzed primarily by TLC and GLC methods outlined and referenced in sections 6.3.1 and 6.3.2.

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# IMIPENEM

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## Acknowledgements

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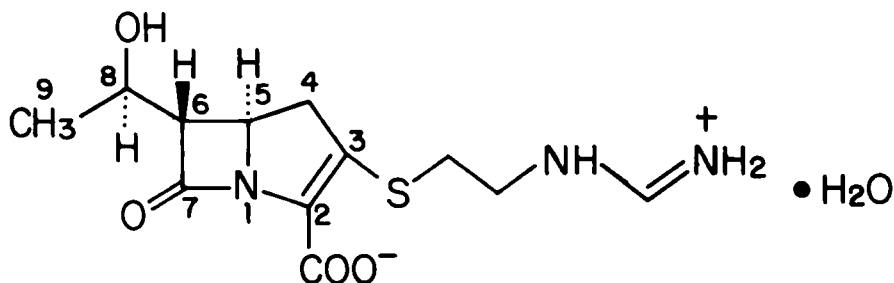
## 1.0 Introduction

Imipenem is a crystalline derivative of thienamycin, a beta-lactam antibiotic whose discovery in cultures of Streptomyces cattleya was reported by J. Kahan, F. Kahan and others of the Merck, Sharp and Dohme Research Laboratories (1). Isolation and properties of thienamycin have been described (1,2,3). The structure of thienamycin and the configuration of the three chiral carbons were established by Albers-Schoenberg, et al. (2). Imipenem is made from thienamycin obtained from fermentation or synthetic processes. It has a high degree of stability in the presence of beta-lactamases, both penicillinases and cephalosporinases, produced by gram-negative and gram-positive bacteria. In vitro, imipenem is active against most strains of Pseudomonas (except P. maltophilia), Streptococcus, Staphylococcus, Bacteroides, Clostridium, Salmonella, Klebsiella, Enterobacteria, Hafnia, Serratia, Proteus, Citrobacter, Nocardes, Actinomyces, and many others. In order to increase its efficacy in urinary tract infections, it is administered with the renal dehydropeptidase inhibitor sodium cilastatin. As the combination it is marketed by Merck Sharp & Dohme as Primaxin®.

## 2.0 Description

Imipenem is a white to off-white or yellowish, crystalline, non-hygroscopic solid.

### 2.1 Structural and Molecular Formulas and Molecular Weight



Molecular Formula:  $C_{12}H_{17}N_3O_4S \cdot H_2O$

Molecular Weight: 317.37

## 2.2 Nomenclature

### 2.21 Chemical Names

[5R-[5 $\alpha$ ,6 $\alpha$ , (R\*)]]-6-(1-hydroxyethyl)-3-[[2-[(iminomethyl)amino]ethyl]thio]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid monohydrate

N-Formimidoylthienamycin monohydrate

### 2.22 Generic (USAN)

Imipenem

### 2.23 Chemical Abstracts Service Registry Numbers

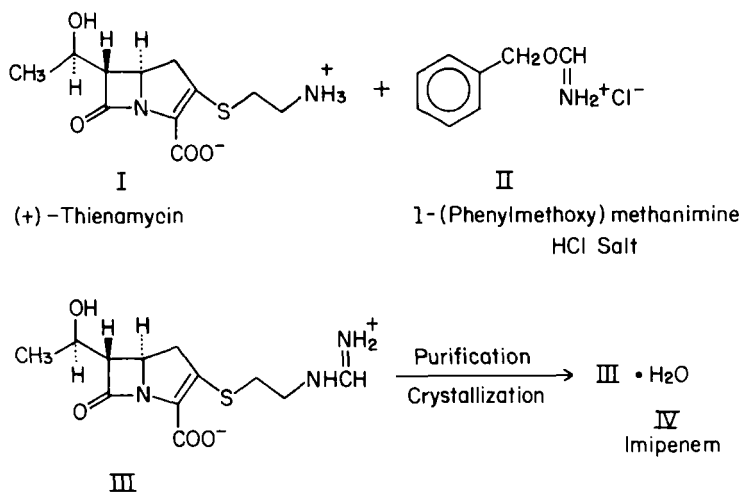
(CAS No.)

74431-23-5 monohydrate

64221-86-9 anhydrous

## 3.0 Synthesis

3.1 Scheme 1. Via thienamycin isolated by absorption chromatography from fermentation broths (4).



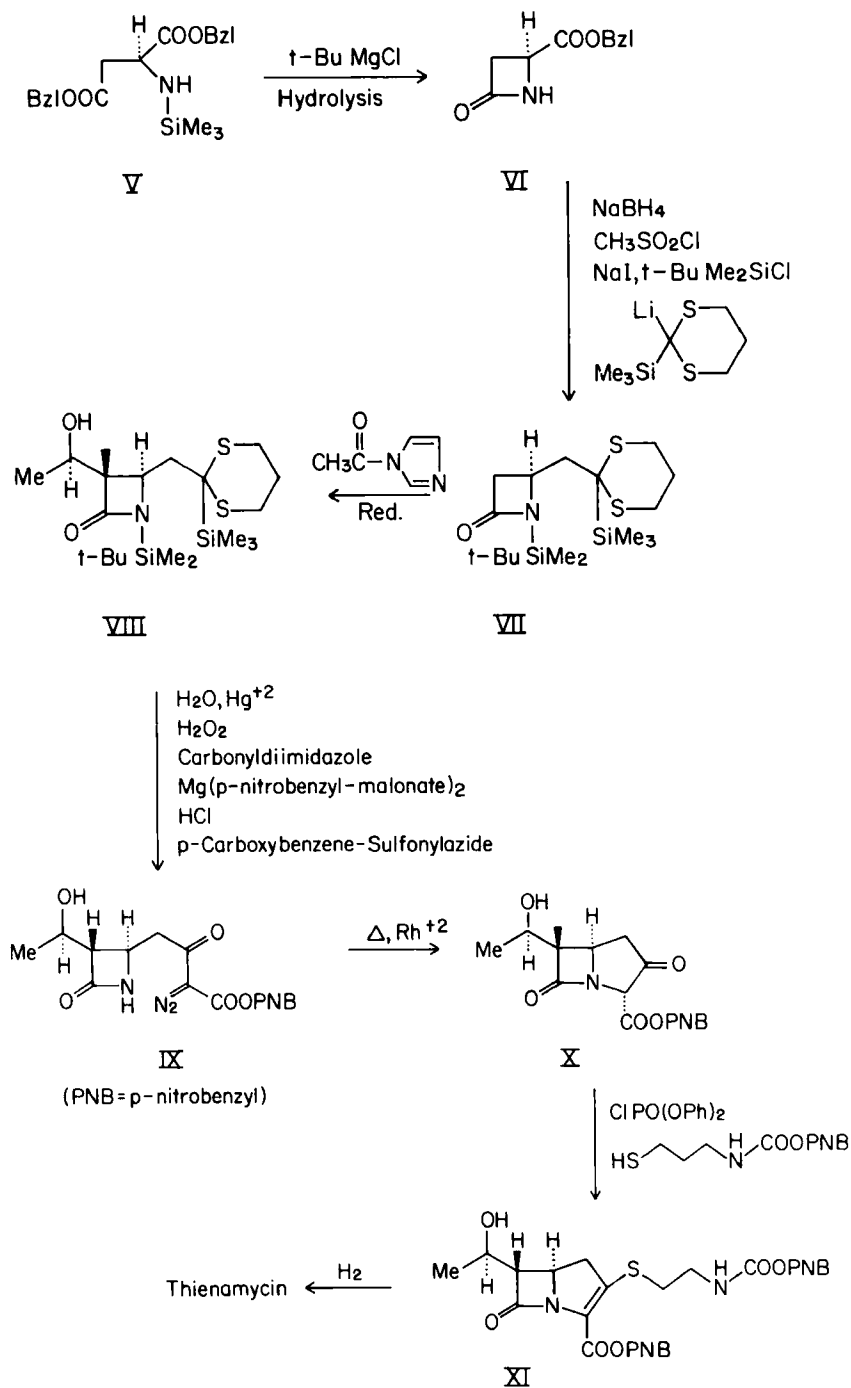
(II is synthesized from benzyl alcohol, formamide and benzoyl chloride).

The formamidine side chain has also been added by reaction of thienamycin with  $\text{MeOCH}=\text{NH}_2^+\text{Cl}^-$  (5).

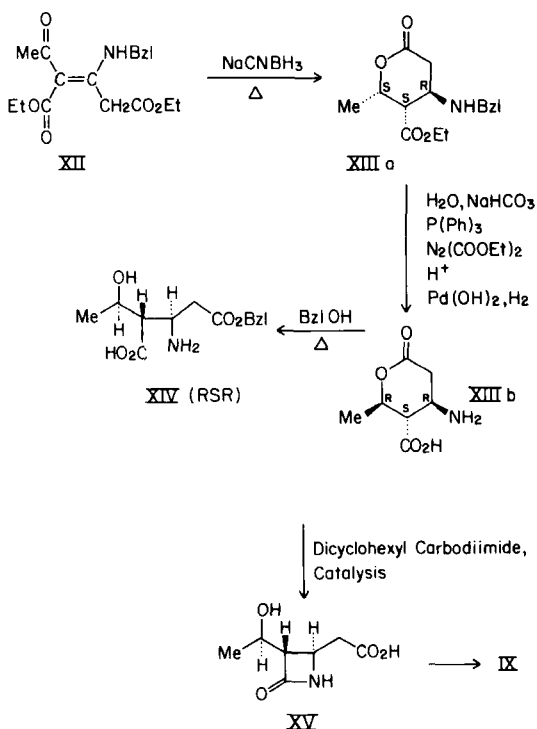
- 3.2 Via total synthesis. The total syntheses of (+)-thienamycin outlined below in schemes 2, 3 and 4 differ in choice of starting materials and in the point along the sequence where cyclization to beta-lactam occurs and where the required RSR configuration of the three chiral carbons is achieved. Synthesis of (+) thienamycin has also been reported with 3(R)-hydroxy-butanoic acid (6) and the methyl ester (7), from penicillin (8) or from D-glucose (9). There are many other approaches to the synthesis of (+)-thienamycin from other and equivalent chiral processes. The following three schemes lead to the diazo ester IX which is easily cyclized in good yield to provide intermediate X for the remaining steps.

- 3.21 Scheme 2. From aspartic acid (10). The N-trimethylsilyl dibenzyl ester (V) is cyclized with Grignard reagent to produce the  $\beta$ -lactam VI. This is treated with the reagents listed to produce, in order, an alcohol, the mesylate ester, the N-protected t-butyl-dimethylsilyl compound and finally the trimethyl silyldithiane derivative VII. This is acylated with N-acetyl-imidazole, then reduced to the desired R(+) isomer VIII. This is converted to IX as shown. Compound IX is heated in toluene or benzene with a rhodium catalyst, producing X. Treatment with diphenyl chlorophosphate and then N-(p-nitro-benzyloxycarbonyl) cysteamine produces XI, which is hydro-generated catalytically to form thienamycin, I.

Compound X can also be converted directly to N-formimidoyl thienamycin by treatment of the diphenylphosphate ester (from  $\text{ClPO}(\text{OPh})_2$ ) with an N-protected-formamidine cysteamine derivative and reduction of the product (11).

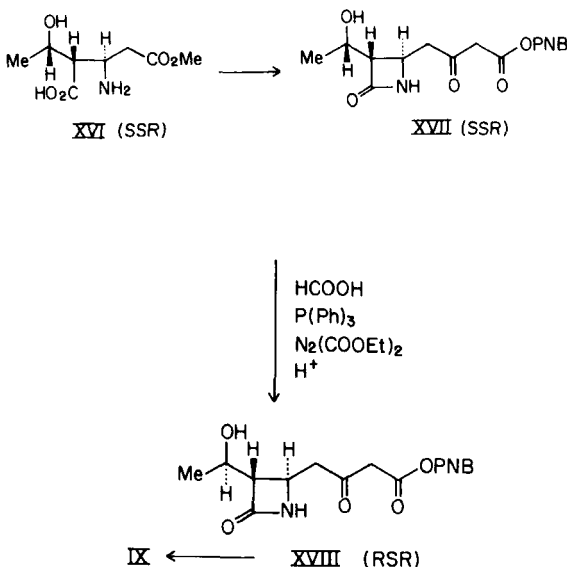


- 3.22 Scheme 3. From acetonedicarboxylate ester (12). Diethyl 1,3-acetonedicarboxylate is converted to XII by treatment with benzylamine and then ketene or acetic anhydride. Reduction and cyclization produce the SSR lactone XIIIa. This is opened by mild hydrolysis and closed again to the RSR configuration by treatment with triphenylphosphine and diethylazodicarboxylate, followed by acid hydrolysis. The product is then catalytically N-debenzylated to the RSR lactone XIIIb which is heated in benzyl alcohol to produce XIV, which retains the desired RSR configuration. Compound XIV is dehydrated with N,N'-dicyclohexylcarbodiimide to produce a  $\beta$ -lactam which is then converted to the free acid, XV. At this point, synthesis proceeds to IX as in Scheme 2 without the dithiane hydrolysis and oxidation via  $H_2O_2$ .





- 3.23 Scheme 4 (13). SSR lactone acid from XIIIa is resolved into isomers with 10-camphorsulfonic acid and debenzylated with hydrogen/palladium. Methanolysis affords XVI, which is cyclized and saponified as in the sequence XIV  $\rightarrow$  XV. Treatment with carbonyldiimide and magnesium p-nitrobenzylmalonate affords XVII. Inversion to the RSR configuration is effected at this point by treatment with formic acid and the reagents used to achieve lactone inversion as in Scheme 3, followed by acid hydrolysis, yielding XVIII. The diazo substituent in IX is provided by p-dodecylbenzenesulfonyl azide, which is safer to use than the carboxyl analog.



In an alternate process (14) similar to Schemes 3 and 4, dimethyl 1,3-acetonedicarboxylate is condensed with (+)- $\alpha$ -methylbenzylamine, which after treatment with ketene or acetic anhydride and reduction yields a lactone analogous to XIIIa which is ultimately converted to the open chain intermediate XVI without the use of 10-camporsulfonic acid as a resolving agent.

#### 4.0 Physical Properties

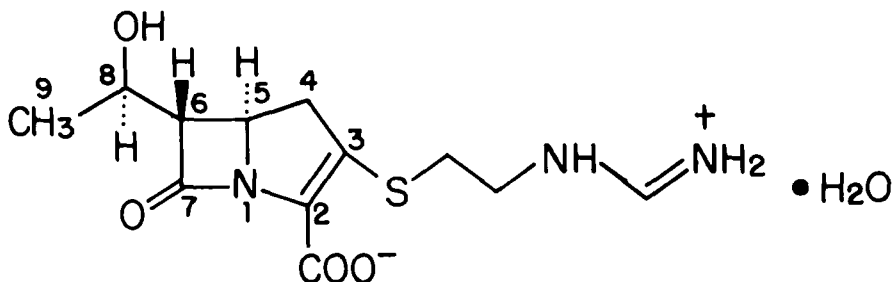
##### 4.1 Spectra

##### 4.12 Ultraviolet Absorbance Spectrum

The ultraviolet absorbance spectrum of imipenem is shown in Figure 1 (15). The spectrum in pH 7.0 phosphate buffer is characterized by a maximum at 298 nm with an  $A_{1\%}^{1\text{cm}}$  value of  $\sim 326$  (anhydrous basis).

##### 4.13 Infrared Absorbance Spectrum

The infrared absorbance spectrum of imipenem (as a mull in mineral oil) is shown in Figure 2 (16). The spectrum is consistent with the structure proposed for imipenem as shown below. Assignments of characteristic bands are listed following the structure.



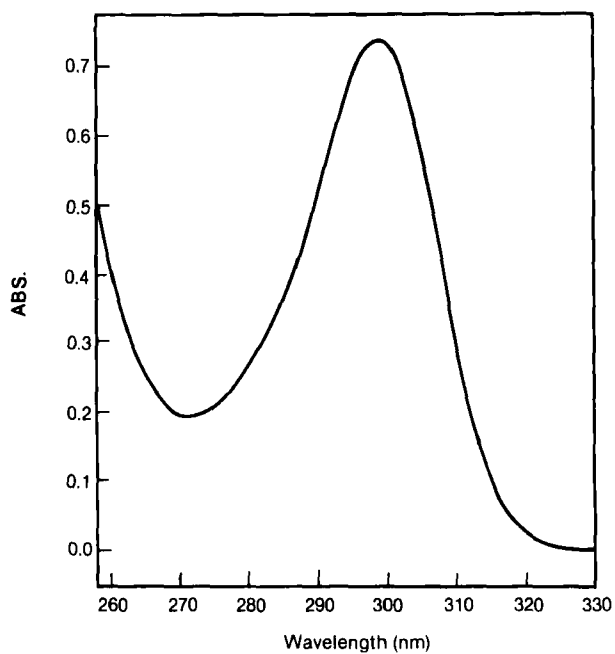


Figure 1

Ultraviolet Absorption Spectrum of Imipenem  
Concentration: 2.4 mg/100 ml Solvent: pH 7 phosphate buffer  
Cary Model 118 Spectrophotometer

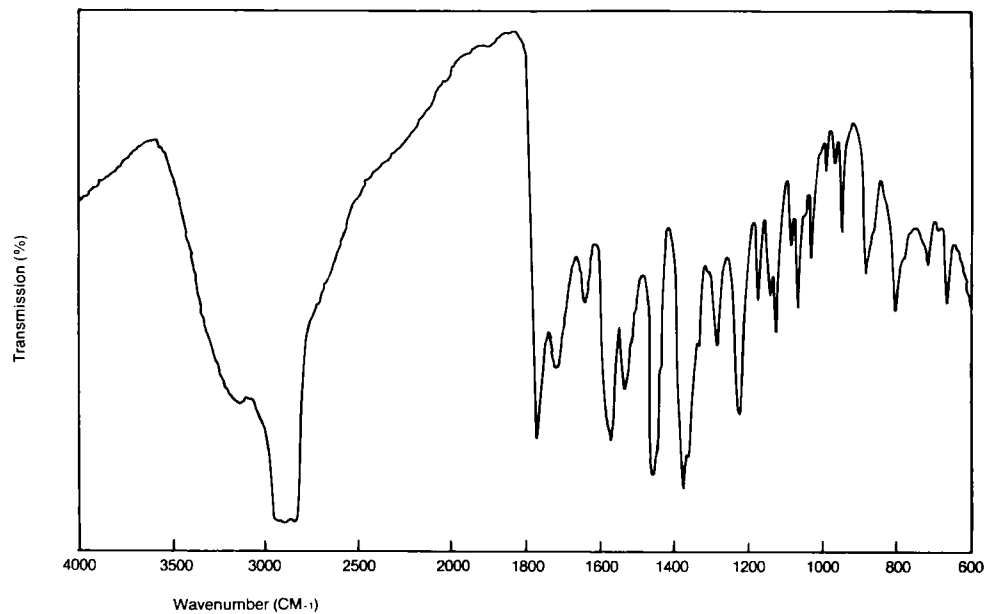


Figure 2  
Infrared Absorption Spectrum of Imipenem  
Mineral Oil  
Perkin-Elmer Model 281-B IR Spectrophotometer

<u>Wave Number (<math>\text{cm}^{-1}</math>)</u>	<u>Assignment</u>
3500-2750	Broad band; Zwitterion and hydrogen bonding
2840-2960	Mineral Oil
1780	Beta-lactam $\text{C}=\text{O}$
1735	Formimidoyl $\text{C}=\text{N}^+$
1655	$\text{H}_2\text{O}$ hydrate
1590	$\text{COO}^-$ asymmetric stretch
1470	Mineral Oil
1390	$\text{COO}^-$ symmetric stretch

Figure 3 shows the spectrum in a KBr disk.

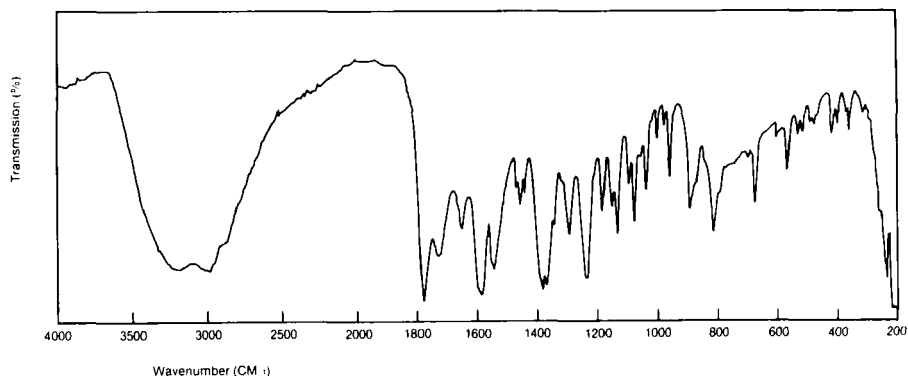
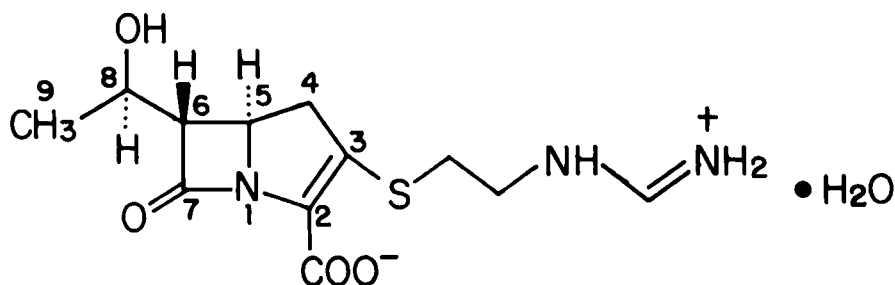


Figure 3  
Infrared Absorption Spectrum of Imipenem  
KBr Disc  
Perkin-Elmer 783 IR Spectrophotometer

#### 4.14 Proton Magnetic Resonance Spectra (17)

The proton NMR spectra shown in Figures 4 and 5 were obtained using a Bruker Instruments Model WM-250 spectrometer (frequency: 250 MHz) and an approximately 2% w/v solution of imipenem in deuterium oxide. The NMR reference (internal) was 4,4-dimethyl-4-silapentanesulfonic acid, sodium salt. Spectral assignments (Table I) refer to the numbered structure.



Chemical shift ranges are given for all multiplets in the spectrum. The complexity of the patterns is due to the presence of a mixture of rotameric conformers (Sections 4.15, 4.9).

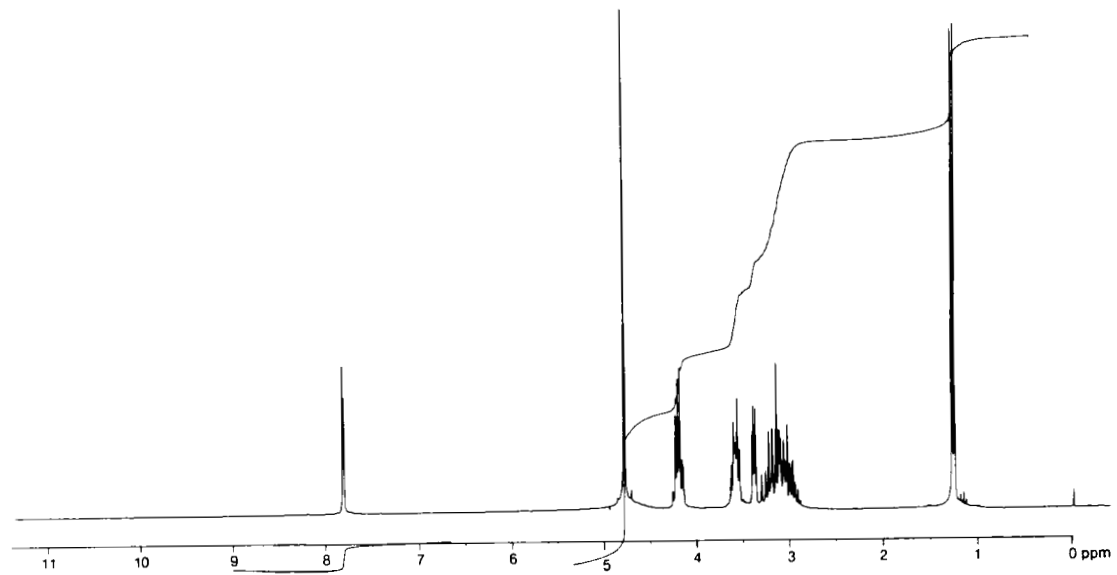


Figure 4  
Proton Magnetic Resonance Spectrum  
of Imipenem

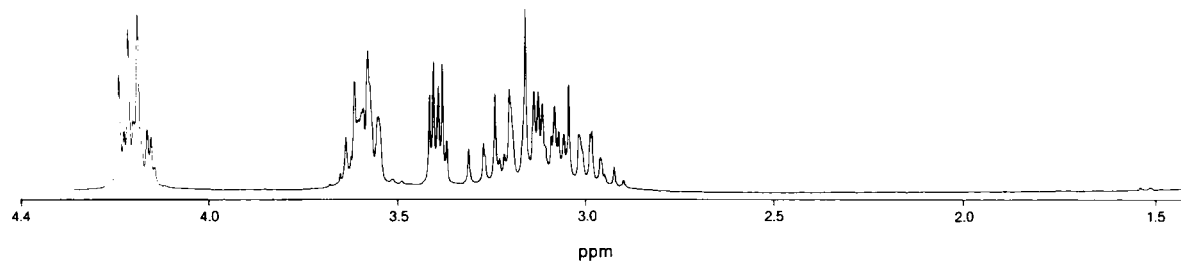


Figure 5  
Proton Magnetic Resonance Spectrum  
of Imipenem  
1.5-4.5 ppm Region Expansion



Table I

Imipenem Proton NMR Assignments

<u>Chemical Shift</u> <u><math>\delta_H</math> in PPM</u>	<u>Multiplicity</u>	<u>Relative No.</u> <u>of Protons</u>	<u>Assignment</u>
7.81	2s	0.9	isomeric $\underline{H}-C=NH_2^+$
4.79		---	Active protons (as HDO)
4.26-4.13	m	1.9	$C_5H, C_8H$
3.67-3.47	m	2.1	$-SCH_2CH_2NH-$
3.42-3.37	m	1.0	$C_6H$
3.31-2.88	m	4.2	$C_4H_2,$ $-SCH_2CH_2NH-$
1.28	d	3.0	$C_9H_3$
1.14	t	---	$CH_3CH_2OH$

(residual solvent in sample)

4.15 Carbon-13 Magnetic Resonance Spectra (18)

The carbon-13 NMR spectra shown in Figures 6 and 7 were obtained on a Varian Associates Model XL-100 spectrometer using a 2% w/v solution of imipenem in deuterium oxide. The NMR reference (internal) was p-dioxane.

Assignments are in Table 2. The C-numbers refer to the same structure as in Section 4.14.

Imipenem exists as a pair of equilibrating formamidine rotamers similar to those commonly seen with secondary formamides (Sec. 4.9).

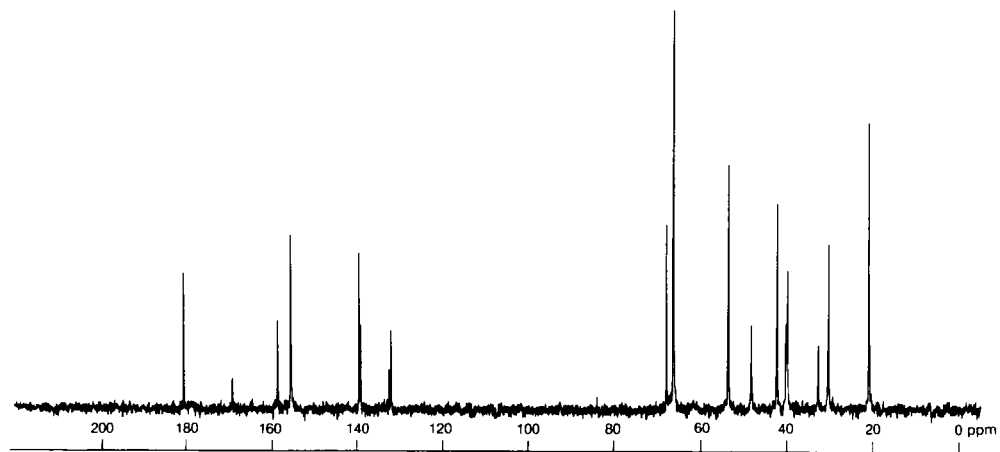


Figure 6  
C-13 NMR Spectrum of Imipenem

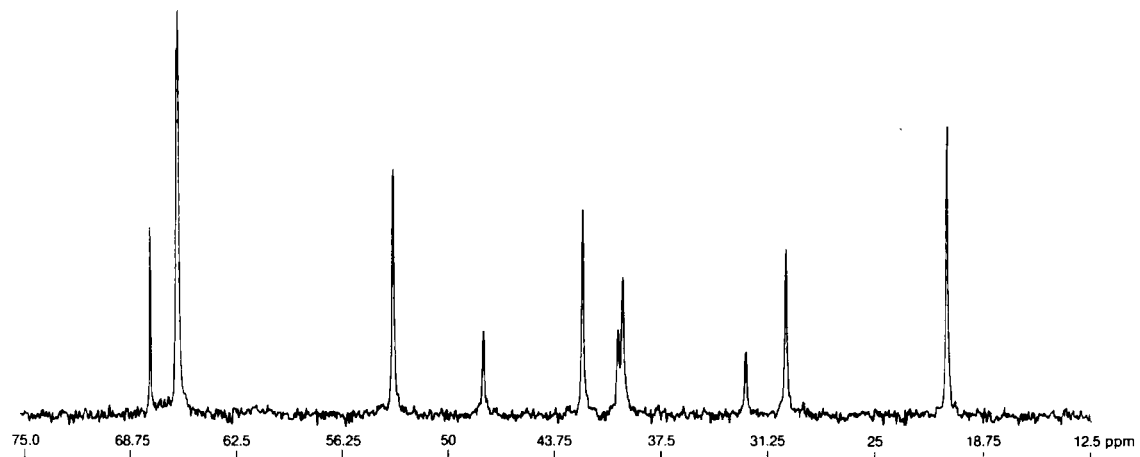


Figure 7  
C-13 NMR Spectrum of Imipenem  
12.5-75 ppm Region Expansion

Signals due to the minor rotameric isomer are given in parentheses wherever they are resolved from the signals of the dominant form.

Table 2

Imipenem C-13, NMR Assignments

<u>Chemical Shift, <math>\delta</math> C, ppm</u>	<u>Assignment</u>
180.4 (180.3)	C <sub>7</sub> (lactam)
169.1	<u>C</u> O <sub>2</sub>
	+
155.3 (158.4)	-NH- <u>C</u> H=NH <sub>2</sub>
139.1 (138.7)	C <sub>3</sub>
131.6 (132.1)	C <sub>2</sub>
65.8	C <sub>6</sub>
65.7	C <sub>8</sub>
53.2	C <sub>5</sub>
42.0 (47.8)	-SCH <sub>2</sub> <u>C</u> H <sub>2</sub> NH
39.7 (39.9)	C <sub>4</sub>
30.2 (32.5)	-SCH <sub>2</sub> CH <sub>2</sub> <u>C</u> H <sub>2</sub> NH
20.9	C <sub>9</sub> ( <u>C</u> H <sub>3</sub> )

4.16 Mass Spectrum (19)

The mass spectrum of imipenem (Figure 8) was obtained with a Finnegan-Mat Model 731 mass spectrometer by negative ion Fast Atom Bombardment using xenon for FAB ionization. (M-H) = 298 was observed, and a prominent fragment at m/e 228 appropriate for side chain (-CH<sub>2</sub>CH<sub>2</sub>NHCH=NH) loss.

4.2 Thermal Behavior

The thermogram of imipenem obtained by differential scanning calorimetry (closed cup, heating rate = 20°C/min) is characterized by a broad endotherm at ~130-140°C. This thermal behavior is attributed to both loss of hydrate water and sample decomposition.

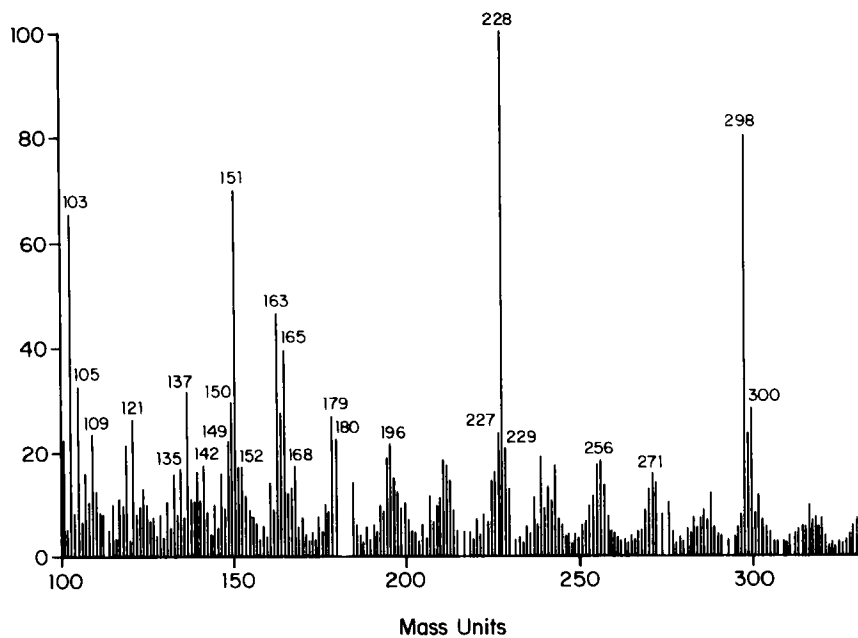


Figure 8  
Mass Spectrum of Imipenem  
Fast Atom Bombardment (negative ion mode)

### 4.3 Solubilities

The solubility data in Table 3 are approximate values obtained at room temperature.

Table 3

#### Solubility of Imipenem

<u>Solvent</u>	<u>Solubility (mg/ml)</u>
Water	10
Methanol	5
Ethanol, 95% (v/v)	0.2
Acetone	<0.1
Dimethylformamide	<0.1
Dimethylsulfoxide	0.3

Water solubility increases to ~22 mg/ml at 50°C and to ~100 mg/ml at 80°C. Table 4 shows water solubility at lower temperatures.

Table 4

#### Equilibrium Solubility of Imipenem (20) in Water

<u>T(°C)</u>	<u>Solubility (mg/ml)</u>	
	<u>Obs.</u>	<u>Calc.*</u>
5	5.1	5.2
11	7.2	6.7
25	10.3	11.3
40	19.3	18.7

\*Calculated from least square line [ $\log(\text{sol.})$  vs.  $1/T$ ]

### 4.4 Dissociation Constants (pKa)

The pKa values for imipenem determined by aqueous acidic/basic potentiometric titration at 25°C are pKa<sub>1</sub> ~3.2 and pKa<sub>2</sub> ~ 9.9 (21).

### 4.5 Partition

Imipenem does not partition into n-octanol or chloroform from aqueous solutions.

#### 4.6 Chirality and Specific Rotation

Imipenem has three chiral centers and is optically active. The specific rotation,  $[\alpha]_D^{25}$  is  $\sim +85^\circ$  ( $c = 0.5$ ,  $0.1M$  pH 7 phosphate buffer) (15).

#### 4.7 Hygroscopicity

Measured at  $25^\circ C$  and at relative humidities of 11 and 76%, imipenem is nonhygroscopic.

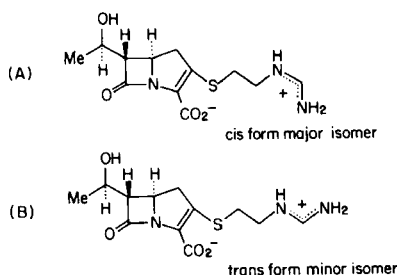
#### 4.8 Crystallinity

Imipenem is crystalline as determined by x-ray powder diffraction. All lots of imipenem prepared by either semi-synthetic modification of natural thienamycin or synthetic procedures have exhibited similar diffraction patterns. The diffraction pattern is shown in Figure 9.

Additional evidence for crystallinity is seen by microscopy. A sample of imipenem examined with the polarizing microscope exhibits birefringence and extinction on revolving the stage of the microscope.

Anhydrous N-formimidoyl thienamycin is amorphous. This solid is hygroscopic and consequently much less stable to ordinary storage.

Imipenem exists in cis- and trans- rotational isomers at the formamidine side chain as shown in (A) and (B).



Single crystal studies indicate that the compound exists in the cis-form (A) in the solid state (22,23). In solution, the compound equilibrates

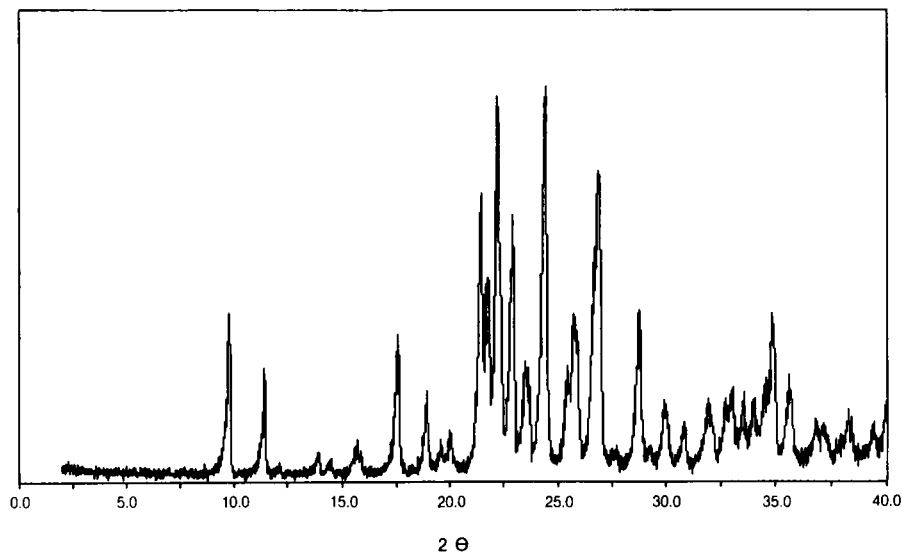


Figure 9  
X-Ray Diffraction Pattern of Imipenem  
Phillips X-Ray Diffractometer



to a mixture of cis- and trans-species. These changes can be monitored by NMR (Section 4.15) and HPLC systems (Sec. 7.52). The single crystal X-ray structure shows the cis-form of the side chain and the network of hydrogen bonds in the crystal lattice (23). The isomers can be separated at low pH (cf. pH 4.5) and isolated. At higher pH the equilibrium is rapid. The isomers owe their stability to the zwitterionic nature of imipenem, which results in partial double bond character between N1 and the C of the formamidine group (23).

## 5.0 Chemical Properties

### 5.1 Solid State Stability

#### 5.11 Photochemical Stability

Imipenem exhibits brownish surface discoloration after exposure to ultraviolet light (20X sunlight) for 24 hours. The compound is not affected by exposure to fluorescent light (1000 ft.-candles) for several weeks.

#### 5.12 Thermal Stability

The compound can be degraded by exposure to high temperatures. A loss of 4-7% occurred after storage at 60° for 16 weeks. Imipenem shows acceptable stability at ambient temperatures; minor losses (ca. 4%) occurred after 2 years at 25°C. The products from solid state degradation have not been identified. It is very likely that the same mode of decomposition observed in aqueous solutions applies in the solid state, i.e., initial cleavage of the beta-lactam followed by formation of multiple degradates.

### 5.2 Solution Stability

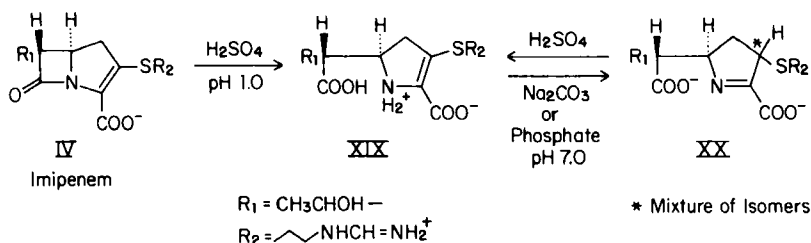
#### 5.21 Solution Kinetics and Compatibility

G. B. Smith has studied the stability of buffered solutions in detail (24). The rate of loss is minimal at pH near 7 and

increases sharply at pH less than 6 or more than 8, as observed in nonnucleophilic buffer systems (MOPS, 3-[N-morpholino] propane-sulfonic acid or MES, 2-[N-morpholino]ethanesulfonic acid). At low concentrations, 1-2 mg/ml (3-6 mM), pseudo-first order kinetics predominates, but second-order reaction becomes significant at higher concentrations. Increasing phosphate concentration hastened the first-order decomposition. On the other hand, oxygen has no effect on the initial loss rate, but the ultimate decomposition products are different. Imipenem is generally less stable than penicillins or cephalosporins in the presence of acids and of phosphate anions (24). Other potential buffers such as tromethamine, lactate, maleate and bicarbonate are also nucleophilic towards the compound. F. Bigley has reported the effects of commonly used IV additives on the solution stability of buffered imipenem and sodium cilastatin (25). Sterile diluents containing sodium lactate, dextrose, mannitol and high concentrations (5%) of sodium bicarbonate significantly reduce the stability of imipenem solutions. Unbuffered aqueous solutions of imipenem decompose quickly due to generation of acidic degradates, which lower the pH to 4.5-5.0. As much as 10% loss can be observed after one hour. However, sodium bicarbonate at 20-40 mg per g of the 1:1 powder mix (with sodium cilastatin) dosage forms provides optimum buffer capacity without compromising shelf life of solutions for IV infusion ( $T_{90\%}$  ca. 10 hr.). Such solutions can be kept at 5°C for 24 hours without significant loss. Chloride does not adversely affect solution stability, and solutions containing up to 5 mg/ml each of imipenem and sodium cilastatin show no appreciable reaction of one compound with the other. Imipenem does not chelate with  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Zn}^{++}$ , or  $\text{Fe}^{+++}$ , but  $\text{Cu}^{++}$  and  $\text{Pb}^{++}$  accelerate solution decomposition (26). Ferrous ion ( $\text{Fe}^{++}$ ) also hastens solution degradation (27).

## 5.22 Mechanism of Solution Degradation

A major product of the first-order solution degradation of imipenem (IV) is an open ring structure which exists as XIX at acid pH and XX at pH 7 or above, as characterized by proton and carbon-13 NMR, UV and FTIR (28).



Structure XX was verified by conducting the reaction in  $\text{D}_2\text{O}/0.115\text{M}$  phosphate at pH 7.0 (28). Proton NMR indicates deuterium exchange at position 3 adjacent to the S of the side chain  $\text{R}_2$ . Structure XX is also the major form of DHP-1-metabolized imipenem, since studies have established the acid form XIX from enzyme-degraded material (29). Air oxidation eventually produces from these compounds other degradates, some of them colored.

The second order reaction is dependent on pH in a manner different from first order as described in Section 5.21 (30). At low pH, the carboxyl of one molecule attacks the beta-lactam of another to produce a dimer. At high pH, the deprotonated formimidoyl of one molecule attacks the beta-lactam of another to produce thienamycin and a 6-amido-N-formyldihydropyrrole derivative. Thienamycin is also produced

by pseudo first-order reaction from imipenem at  $\text{pH} \geq 9$ .

The second order products react further to yield a variety of compounds.

## 6.0 Microbiology

Imipenem, like other beta-lactam antibiotics, is a specific inhibitor of bacterial cell wall synthesis. Reduction in activity with high cell densities is less than with other beta-lactam antibiotics, and a very low incidence of cross-resistance to imipenem has been encountered (31). References on comparison of minimum inhibitory concentrations (MIC) of imipenem with those of other antibiotics (32-55) show that the compound generally has a wider range of activity against most gram-negative and gram-positive bacteria. For example, the  $\text{MIC}_{90}$  (concentration necessary to inhibit 90% of the colonies in a given study) against *Staphylococcus aureus* is  $< 0.06 \mu\text{g/ml}$ , against *Escherichia coli*,  $< 0.31 \mu\text{g/ml}$ , against *Pseudomonas aeruginosa*,  $< 4.9 \mu\text{g/ml}$  and against *Bacteroides fragilis*,  $< 0.36 \mu\text{g/ml}$ .

## 7.0 Biopharmaceutics

### 7.1 Pharmacokinetics

Administered by intravenous infusion (20 min.) with an equal dose of sodium cilastatin, imipenem shows peak levels of around  $17 \mu\text{g/ml}$  for a 250 mg dose,  $39 \mu\text{g/ml}$  for a 500 mg dose and  $66 \mu\text{g/ml}$  for a 1000 mg dose. It exhibits a plasma half life of approximately one hour (56-59, 63). The disposition of imipenem is adequately described by a two-compartment open pharmacokinetic model with elimination occurring from the central compartment only (60). Plasma clearance is around 180-210 ml/min and renal clearance 120-135 ml/min if cilastatin is present (56, 60, 63).

Imipenem and its metabolites are excreted primarily through the kidney and minimally through the bile (29,61).

Due to the activity of DHP-1, urinary recovery of imipenem is low, between 6% and 40% of the administered dose, when cilastatin, the DHP-1 inhibitor is absent (57). With inhibitor present, the recovery is 60-80% (57,59,61). Probenecid, which has been used to prolong blood levels of penicillins, is without significant effect on plasma half-life or urinary recovery of imipenem (58,63). Renal impairment slows elimination (59). Multiple dose studies have shown no evidence that imipenem accumulates in the blood stream (58, 60) in the absence of renal impairment.

## 7.2 Metabolism

When imipenem is administered alone, much of the metabolism has been shown to occur in the brush border cell region at the luminal surface of the proximal kidney tubule, where the compound is decomposed by the renal dipeptidase, DHP-1 (29,62). When imipenem is administered with cilastatin, which totally inhibits its renal metabolism, a 30% non-renal metabolite component of its total body clearance has been demonstrated (57, 60, 61). Imipenem metabolism in the circulating plasma is not affected by the presence of cilastatin (29). The primary metabolite is the same open-ring compound which results from in-vitro hydrolysis at  $\text{pH} \geq 7.0$  (29).

## 8.0 Analytical

### 8.1 Elemental Analysis

Reference Lot L-638,596-01D360

$\text{C}_{12}\text{H}_{17}\text{N}_3\text{O}_4\text{S} \cdot \text{H}_2\text{O}$

	<u>Calculated</u>	<u>Found</u>
Carbon	45.41	45.70
Hydrogen	6.05	6.05
Nitrogen	13.24	13.05
Sulfur	10.10	10.07

Corrected for weight loss by vacuum drying (thermogravimetric analysis) to monohydrate basis.

## 8.2 Water Content

Karl Fischer reagent reacts with imipenem and thus cannot be used to determine water content the way it can be with some other beta-lactam antibiotics. Total moisture content can be determined by vacuum thermogravimetric analysis (TGA), in which weight loss is recorded during programming to a pre-determined temperature. Conventional vacuum drying at 60°C only determines weight loss due to unbound water. Higher temperatures (90-100°C) remove some but not all of the bound water.

## 8.3 Ultraviolet (UV) Spectrophotometry

The intact compound can be determined by its absorbance near its maximum at 298 nm, absorptivity ( $A\%$ ) = 309; absorptivity ( $\epsilon$ ) = 9807, in pH 7.0 buffer ("as is"; on the anhydrous basis,  $A\%$  = 326,  $\epsilon$  = 10347).

## 8.4 Spectrophotometric Method with Hydroxylamine

The method is essentially that reported initially for analysis of thienamycin (3) as adapted for N-formimidoyl thienamycin (64). Cleavage of the  $\beta$ -lactam ring destroys the UV chromophore responsible for the  $\lambda$  max near 298 nm. If this occurs in the presence of oxygen (air), products may form which also absorb in this region. Treatment with hydroxylamine HCl in aqueous solutions buffered at pH 7.0 rapidly degrades imipenem to a substance not possessing absorbance at 298 nm. It does not affect decomposition products already present which may show absorbance. A suitable stability-indicating assay is thus based on the difference in absorbance measured before and after treatment with hydroxylamine HCl. Typical applications have been described (2, 24, 64). The method has been shown to be essentially equivalent to both microbiological and chromatographic procedures (65). It has been adapted for use in flow-injection analysis systems (66). There is no interference from cilastatin, which is commonly present in the dosage forms. The quenching of UV absorbance is faster with

increasing hydroxylamine concentration. Non-nucleophilic buffers such as MOPS (N-morpholino-propanesulfonic acid) or MES (N-morpholinoethanesulfonic acid) may be preferred in order to avoid losses of imipenem which may occur on standing in phosphate or other nucleophilic buffers. The method is equally applicable to imipenem and thienamycin but does not distinguish between the two.

## 8.5 Chromatography

### 8.51 Thin-Layer Chromatography

Imipenem can be chromatographed on silica gel using the following two solvent systems:

#### System I

n-butanol/acetic acid/water/toluene/  
acetone, equal parts

#### System II

n-butanol/acetic acid/water 5:2:3

A typical  $R_f$  of imipenem in these systems is 0.4-0.5. Cilastatin, if present, develops with  $R_f$  0.7-0.8. Commercial plates may vary in adsorption characteristics. Fluorescent plates are used. Visualization of spots using any of the adsorbants and developing systems is by UV fluorescence quench or iodine vapor staining. Thin layer chromatography of imipenem is not recommended as a quantitative method since some decomposition is apt to occur during chromatography.

### 8.52 Liquid Chromatography

Table 5 lists high performance (high pressure) liquid chromatographic (HPLC) systems which have been used for analysis of imipenem. System III, VI and IX are of value in determining imipenem and cilastatin simultaneously (System III, Figure 10).

Table 5. HP Chromatographic Systems for Imipenem

<u>System No.</u>	<u>Column</u>	<u>Mobile Phase</u>	<u><math>\lambda_{det}</math> nm</u>	<u>Ref.</u>
I	Lichrosorb RP18	0.001M $\text{KH}_2\text{PO}_4$ , pH 6.8	300	67
II	Whatman SAX (strong anion exch.)	0.004M Tromethamine	300	68
III	Lichrosorb RP-8	0.004M 'MOPS', pH 7.0 0.2% $\text{C}_6\text{H}_{13}\text{SO}_3\text{Na}$	250	69
IV	Supelco $\text{C}_{18}$ , 5 $\mu\text{m}$	A. 0.025M $\text{C}_6\text{H}_{13}\text{SO}_3\text{Na}$ in 0.1M $\text{NH}_4\text{H}_2\text{PO}_4$ , (pH 4.5) 98% B. Acetonitrile 2%	300	67
V	Chromegabond $\text{C}_{18}$ (Lichrosorb)	Gradient A. 0.001M $\text{KH}_2\text{PO}_4$ , pH 6.8 5 min. B. Acetonitrile, 30 min. to 50%	210	67
VI	Lichrosorb RP8	$\text{H}_2\text{O}$ :0.1M 'MOPS':MeOH 935:30:35	250	70
VII	Lichrosorb RP8	A. 0.003M 'MOPS', pH 7.0, 97% B. Methanol, 3%	250	71
VIII	$\text{C}_{18}$ microBondapak	0.01M $\text{KH}_2\text{PO}_4$ , pH 7.0	298	64
IX	$\text{C}_{18}$ microBondapak	A. 0.002M 'MOPS', pH 7.0, 95% B. Methanol, 5%	298	72
X	$\text{C}_{18}$ microBondapak	'PIC' A, pH 7.0 20 ml/l (0.005M)	215	73



<u>System No.</u>	<u>Column</u>	<u>Mobile Phase</u>	<u><math>\lambda</math> det nm</u>	<u>Ref.</u>
XI	C <sub>8</sub> or C <sub>18</sub>	0.001M phosphate, pH 6.8 with 2 g/l C <sub>6</sub> H <sub>13</sub> SO <sub>3</sub> Na	254	74
XII	Zorbax ODS	0.4% Triethylamine in 0.05M KH <sub>2</sub> PO <sub>4</sub> , pH 7		30
XIII	Plasma: Hypersil ODS Lichrosorb C <sub>18</sub> Guard Col. Urine: Altex 10 $\mu$ m CX cation exch. Partisil 10 $\mu$ m Guard Column	0.2M Borate, pH 7.2  same	313/intact 214/open ring same	75
XIV	Micropak MCH 10 RP Vytak 40 $\mu$ m guard column	93% H <sub>2</sub> O, 7% MeOH	300	76
XV	C <sub>18</sub> microBondapak	Plasma: 'PIC' A 0.05M (tetrabutyl ammonium phosphate) pH 7.1 Urine: 0.1M (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> , pH 4.2	313	29
XVI	(Preparative) Whatman Partisil 10 ODS-2 Magnum-20	Gradient H <sub>2</sub> O/MeOH 0-20% MeOH	295, 220	30
XVII	Waters Radial-pak A	Phosphate buffer, 0.01M, pH 7.0	280	77
XVIII	Partisil PKS 10/25 PAC Whatman Pell. PAC Guard Column	A. Phosphate buffer, 0.05M, pH 7.0, 30% B. Acetonitrile, 70%	254	78

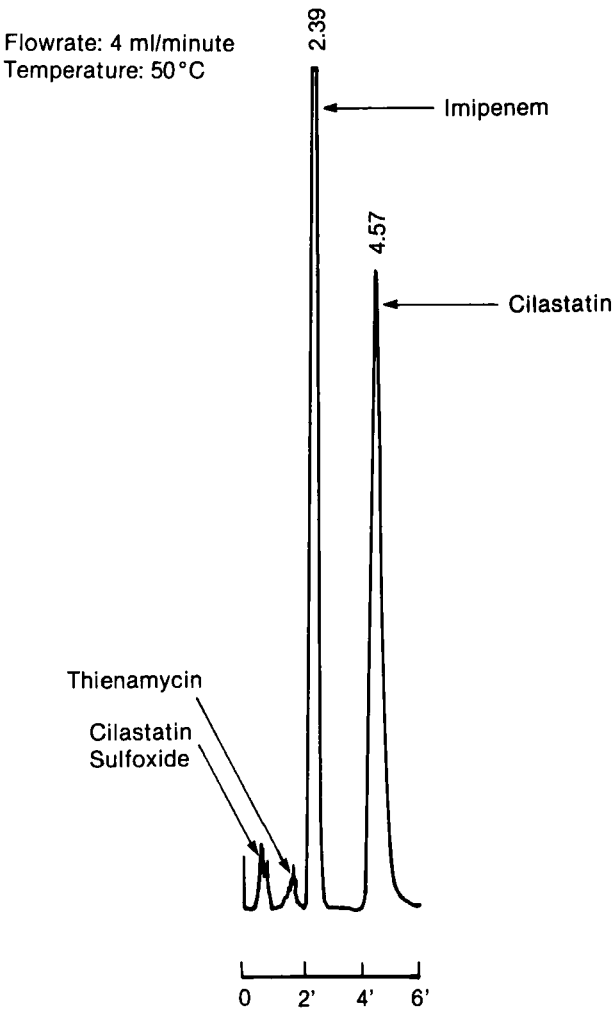


Figure 10-System III  
HPLC Chromatogram of Imipenem and Cilastatin

System IV separates formamidine rotamers (Figure 11), and a gradient chromatogram using System V is illustrated in Figure 12. Systems XIII-XV have been reported for analysis of biological fluids and XVI has been used as a preparative system. MOPS is N-morpholinopropanesulfonic acid.

## 8.6 Microbiological

### 8.61 Preservation of Solutions for Microbiological Testing

It is sometimes desirable to store imipenem solutions until a convenient time for assay. The solutions are unstable and freezing of simple aqueous solutions results in unacceptable loss (20% or more). However, imipenem may be preserved for at least six months at  $-80^{\circ}\text{C}$  in 0.5M MOPS solutions containing 25% ethylene glycol (58, 60, 63).

### 8.62 Process Materials and Dosage Forms (79)

The activity of imipenem is measured by a zone inhibition method. The sample is diluted to approximately 1 mcg/ml with dilute (0.05M) MOPS buffer (pH 7.0). The standard (500 mcg/ml) is diluted to about 0.25, 0.5, 1.0 and 2.0 mcg/ml with 0.05M MOPS. The test organism is MB32, Bacillus subtilis, (ATCC #12432) in a spore suspension containing 10 CFU and Brain Heart Infusion Agar for the sporulation medium. The plate medium contains 5 ml of spore suspension in one liter of Mueller-Hinton agar. Disks impregnated with test solution are put onto plates containing 10 ml of the hardened seeded medium and incubated overnight at  $37^{\circ}\text{C}$ . A Bausch & Lomb Imager analyzer, Fisher-Lilly Zone Reader or other suitable apparatus is used to read and calculate sample potency. The sensitivity of the method using B. subtilis is about 0.3 mcg/ml (60). Known metabolites and degradates do not interfere (60).

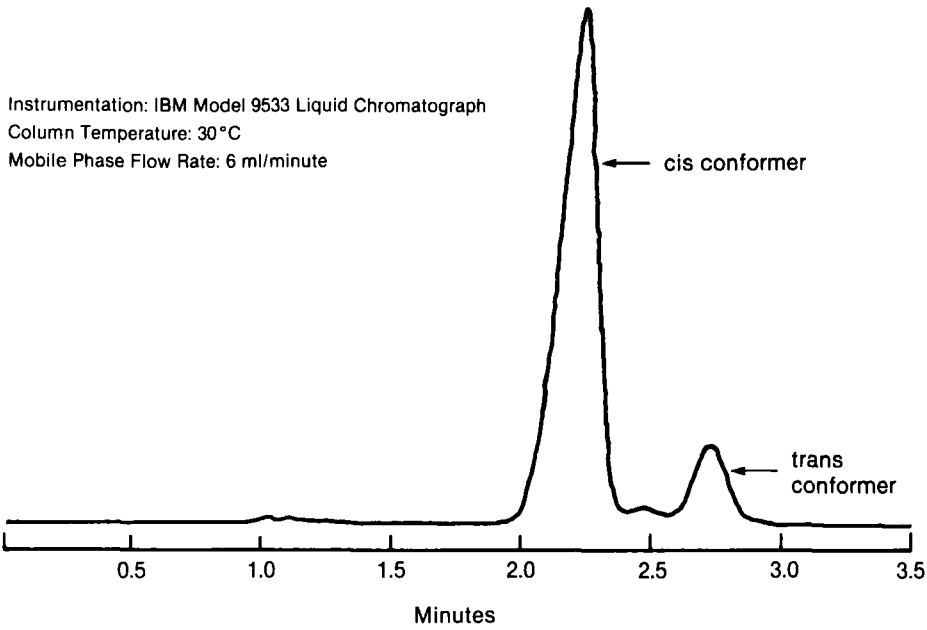


Figure 11-System IV  
HPLC Chromatogram of Imipenem Conformers Separation

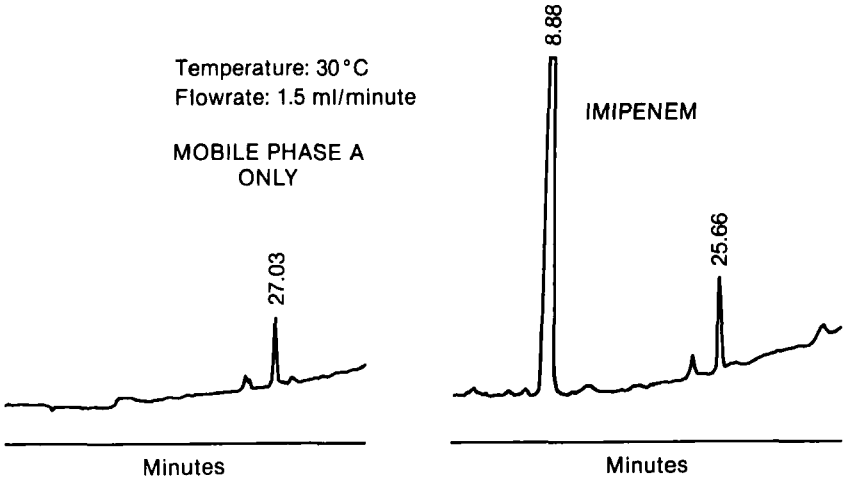


Figure 12-System V (Gradient Elution)  
HPLC Chromatogram of Imipenem

### 8.63 Biological Fluids

Microbiological analysis by agar plate disk-diffusion in serum, plasma and urine has been reported using similar techniques (60). Mueller-Hinton agar is the plate medium and the test organism in MB32, B. subtilis.

### 8.64 Susceptibility Tests

H. Kropp (80) and others reported on microbiological techniques for evaluating susceptibility of a variety of organisms (80, 81, 82, 83). The majority of studies used a microtiter method which records the concentration at which turbidity fails to occur in a test preparation of the antibiotic inoculated with the test organism (80, 81, 82). Other methods used were disk diffusion (78) and a plate method (83).

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# IOPAMIDOL

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## 1. INTRODUCTION

### 1.1 Foreword

Iopamidol is an injectable iodinated contrast agent for angiography, excretory urography, and myelography. It belongs to the class of non-ionic triiodinated benzoic acid derivatives with 3 iodine atoms per particle in solution ( ratio 3 contrast media).

### 1.2 History

With the aim of lowering chemotoxicity and improving heat stability of non-ionic molecules a series of hydroxyalkylamides of 5-( $\alpha$ -hydroxyacyl) amino-2, 4,6 triiodoisophthalic acids was synthesized in the Research Laboratories of Bracco Industria Chimica, Milan Italy, in the early 70's.

Shielding the hydrophobic iodine atoms with highly hydrophilic substituents afforded compounds with promising characteristics. Especially the molecule coded B 15000, later to be named Iopamidol, was deemed worthy of preclinical and clinical development and patent protection (1,2,3,4).

Favourable physico-chemical properties, stability to heat sterilization of the injectable solution , and excellent tolerability allowed broad diagnostic indications for Iopamidol such as lumbar and cervical myelography, cerebral angiography, peripheral arteriography and venography, angiocardiology, coronary arteriography, aortography, selective visceral angiography, CT enhancement, digital subtraction angiography, excretory urography and arthrography.

Updated preclinical and clinical experiences were reported during a worldwide Symposium on Iopamidol, held at Fort Lauderdale in early 1983 (5).

Iopamidol was introduced on the Italian market in September 1981 and soon afterwards in other European countries, whereas U.S.introduction followed at the beginning of 1986.

### 1.3 Therapeutic category

Diagnostic aid

## 2. DESCRIPTION

## 2.1 Nomenclature

### 2.1.1 Chemical Names

1,3-Benzenedicarboxamide-N,N'-bis [2-hydroxy-1-(hydroxymethyl) ethyl]-5-[2-hydroxy-1-oxopropyl)-amino]-2,4,6-triiodo-, (S)-

N,N'bis(1,3-dihydroxy-2-propyl)-5-L-lactoylamino-2,4,6-triiodoisophthalamide.

(S)-N,N'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodo-5-lactamidoisophthalamide

CAS : 60166-93-0

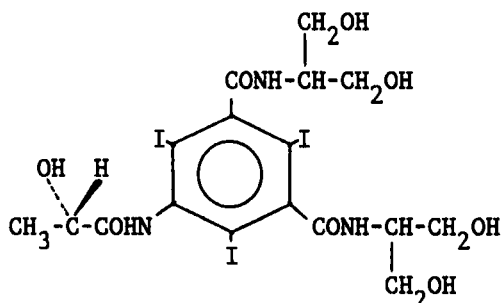
### 2.1.2 Generic Name

Iopamidol (USAN, INN, BAN)

### 2.1.3 Trade Names

Iopamiro	(Bracco)
Isovue	(Squibb)
Niopam	(Merck, UK)
Solutrast	(Byk Gulden)
Iopamiron	(Schering)

## 2.2 Formula, Molecular weight and Iodine Content



$C_{17}H_{22}I_3N_3O_8$

Mol.wt = 777.1

Organically bound iodine : 49.00%

## 2.3 Appearance, Color, Odor and Taste

White crystalline powder, practically odorless,

with a slightly bitter taste (6).

### 3. PHYSICAL PROPERTIES

#### 3.1 Spectra

##### 3.1.1 Ultraviolet Spectrum

The ultraviolet spectrum of Iopamidol (Bracco Working Standard) was determined in water, methanol and borate buffer pH = 9, with a Cary mod.219 spectrophotometer.

The UV spectrum of Iopamidol in water is shown in Fig.1 and some spectral data are presented in Table 1.

Table 1  
UV spectral data

Solvent	$\lambda_{\max}$ (nm)	log $\epsilon$ max	$E^{1\%}_{1\text{cm}}$ ( $\lambda_{\max}$ )		
			mean	s.d.	n
Water	242	4.46	371	0.46	10
Methanol	241	4.47	382	0.61	10
Borate buffer	242	-	380	-	-

##### 3.1.2 Infrared Spectrum

The Infrared spectrum of anhydrous Iopamidol (Working Standard sample) is shown in Fig.2.

The spectrum was obtained on a 0.3% dispersion in KBr with a Perkin Elmer mod.882 Spectrophotometer. Spectral assignments for principal absorption bands given in Table 2 are consistent with the proposed structure.

Table 2  
IR spectral data for anhydrous Iopamidol

Wavenumber ( $\text{cm}^{-1}$ )	Assignment (s)
3380	$\nu\text{OH}$
3240, 3060	$\nu\text{NH}$ , sec.amide
2940, 2880	$\nu\text{CH}$ , aliphatic
1630	$\nu\text{C=O}$ , amide 1st band



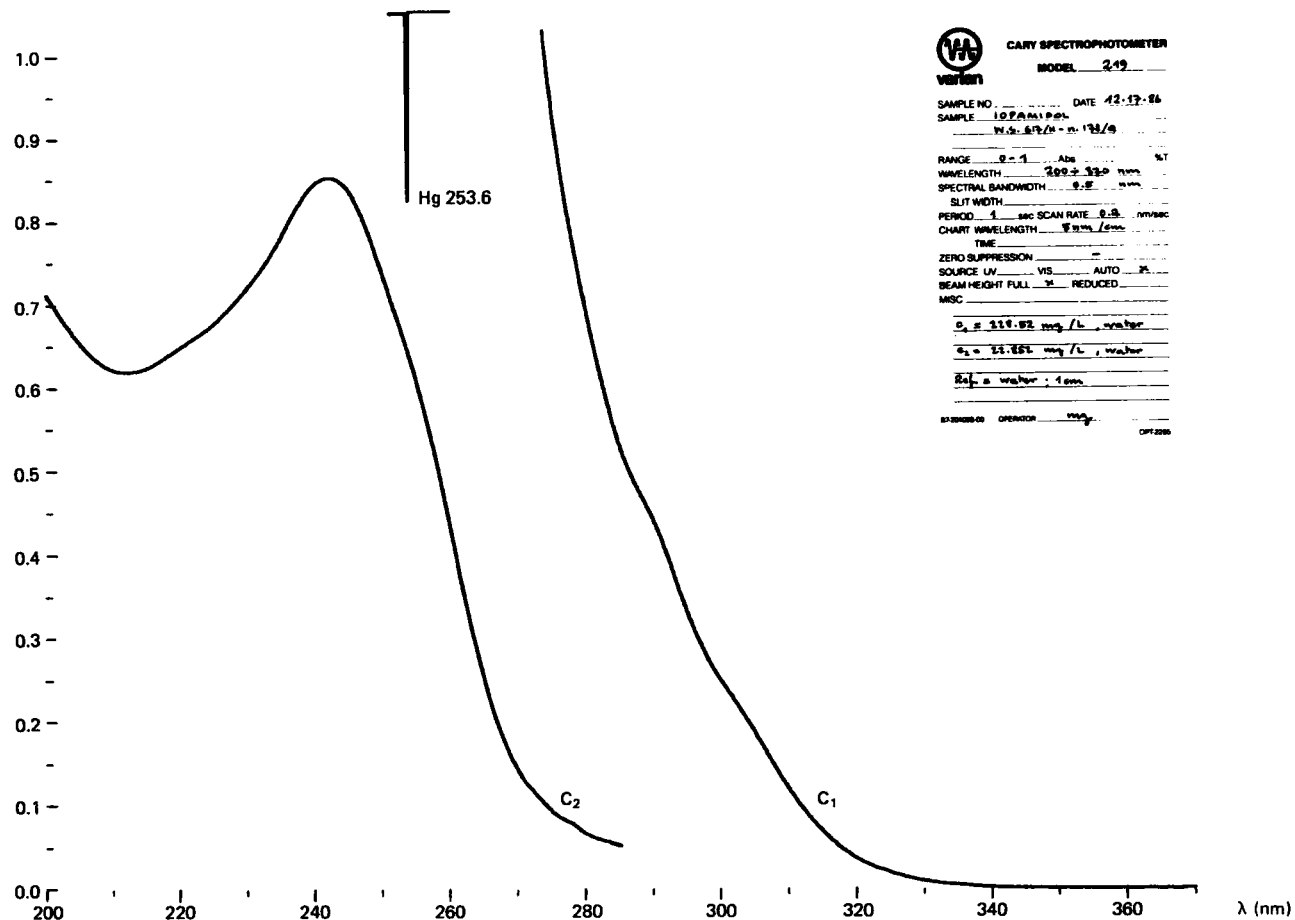


Fig. 1 - Ultraviolet spectrum of Iopamidol in water

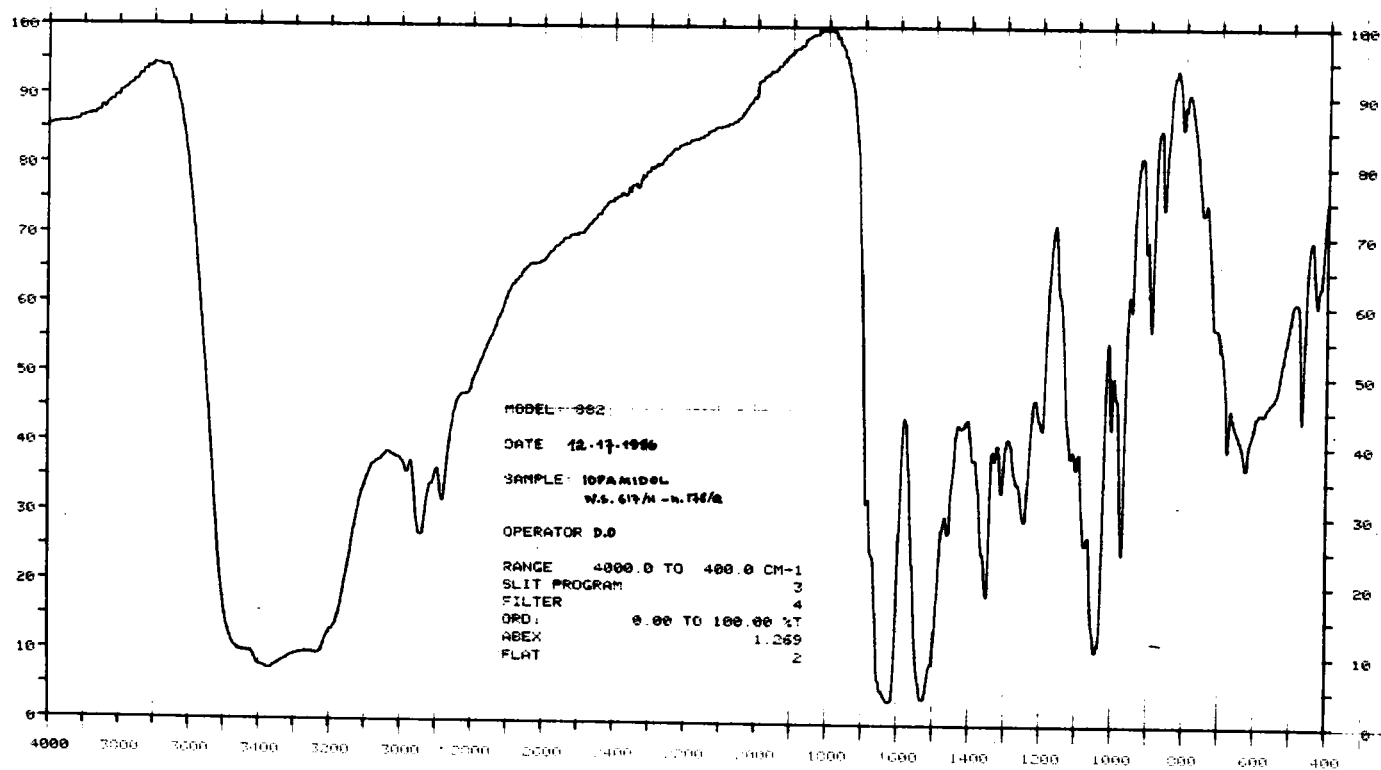


Fig. 2 - Infrared spectrum of Iopamidol in KBr

Wavenumber (cm <sup>-1</sup> )	Assignment (s)
1530	$\delta$ NH+ $\nu$ CN, amide 2nd band
1350	$\nu$ CN+ $\delta$ NH, amide 3rd band
1245	$\delta$ OH
1100	$\nu$ C-O, primary alcohol
1045	$\nu$ C-O, secondary alcohol
970	
890	
680	
625	
470	

The two hydrated forms of Iopamidol (see 3.2.1) gave IR spectra with absorption bands that, although consistent with the structure of the product, showed significant differences between each other and the anhydrous form mainly in the region between 1700 and 800 cm<sup>-1</sup>.

### 3.1.3 Nuclear Magnetic Resonance Spectra

Numerous isomers are predictable to be present at the equilibrium, due to the possible hindered rotations around the aryl-CO, aryl-N, and N-CO bonds. The detectability of ambient temperature isomers has been proved by Bradamante et al. (7): it has been shown by <sup>1</sup>H and <sup>13</sup>C NMR analyses that different free energies of activation pertain to the various hindered rotations, allowing discrimination of the effects.

Variable temperature experiments in DMSO-d<sub>6</sub> and D<sub>2</sub>O solvents (.04 M) indicate that :

- isomers derived from the hindrance of rotation around the aryl-CO bond (syn and anti) characterized by whether or not the C=O double bonds point towards the same direction relative to the plane of the benzene ring, are present in the ratio of 1:1;
- isomers derived from CO-N hindered rotation in the isophthalic carboxamido moiety (E and Z) are present in the ratio of 1:1;
- rotation around the aryl-N bond is fast on the NMR time scale;
- a conformational preference for the endo isomer is detectable in the case of isomers derived from the CO-N hindered rotation in the anilido moiety (endo and exo).

3.1.3.1  $^1\text{H}$ -NMR

$^1\text{H}$  NMR spectra of Iopamidol (Bracco Working Standard) were recorded in  $\text{DMSO-d}_6$  and  $\text{D}_2\text{O}$  solutions with a Bruker AC-200 Spectrometer operating at 200 MHz (8).

Chemical shifts and assignments are reported in Tables 3 and 4, while Figures 3 and 4 show the normal spectrum in DMSO and the 2D spectrum (COSY-90) respectively, further confirming the multiplicities observed and their assignments.

Table 3

$^1\text{H}$ -NMR data (200 MHz) in  $\text{DMSO-d}_6$

Chemical shift $\delta_{\text{H}}$ (ppm,TMS)	Multiplicity	nr.protons	Assignments (s)
9.69	s	1 exch.	$\phi$ -NHCO
8.18 } 7.62 }	b,m b,m	2 exch.	2 $\phi$ -CONH
5.65	m	1 exch.	$\text{CH}_3$ -CH( <u>OH</u> )
4.68	b,t	1 exch.	4 -CH <sub>2</sub> <u>OH</u>
4.52	b,t	3 exch.	
4.18	m	1	$\text{CH}_3$ - <u>CH</u> (OH)
3.87	b,m	2	2 - <u>CH</u> -N
3.7-3.4	m	8	4- <u>CH</u> <sub>2</sub> OH
1.39	d	3	<u>CH</u> <sub>3</sub> -CH(OH)

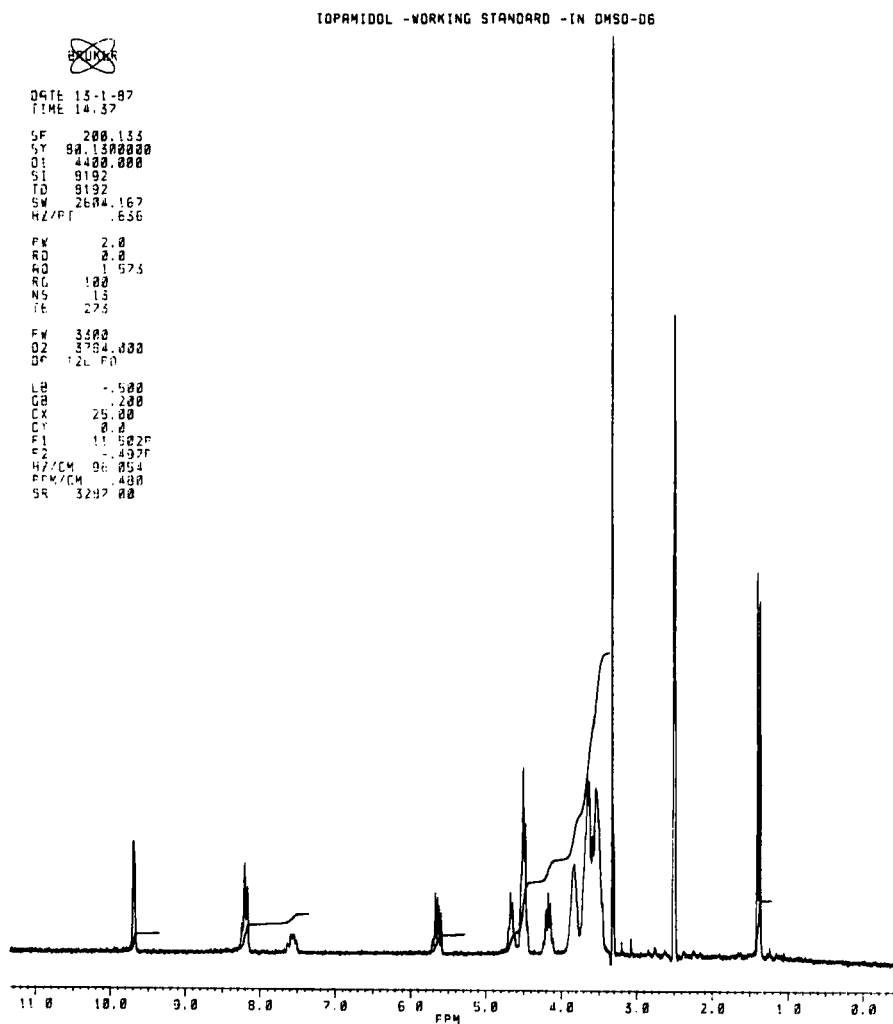


Fig. 3 -  $^1\text{H}$ -NMR spectrum of Iopamidol in DMSO

Fig. 4 - 2D-COSY  $^1\text{H}$ -NMR spectrum of Iopamidol in DMSO

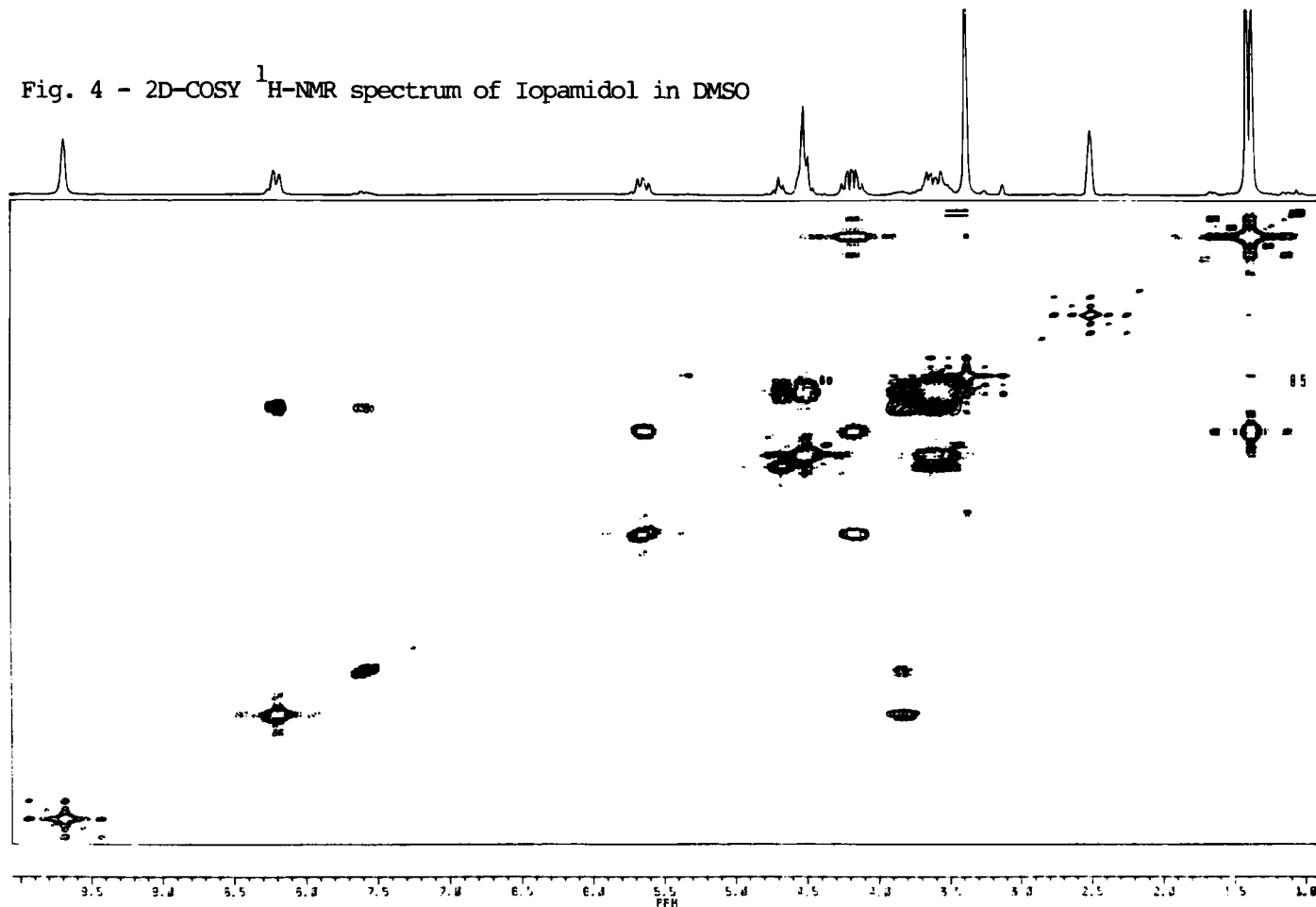


Table 4

<sup>1</sup>H-NMR data (200 MHz) in D<sub>2</sub>O

Chemical shift δ <sub>H</sub> (ppm) (*)	Multiplicity	nr.protons	Assignment(s)
4.49	q	1	CH <sub>3</sub> - <u>CH</u> (OD)
4.16	qui	2	2- <u>CH</u> -N
3.82	d	8	4- <u>CH</u> <sub>2</sub> OD
1.55	d	3	<u>CH</u> <sub>3</sub> -CH(OD)

(\*) corrected for TMS

3.1.3.2 <sup>13</sup>C-NMR

<sup>13</sup>C-NMR spectrum of Iopamidol (Bracco Working Standard) shown in Figure 5 was recorded in DMSO-d<sub>6</sub> solution with a Bruker AC-200 spectrometer at 50 MHz (8). Chemical shifts and assignments are reported in Table 5.

3.1.4 Mass Spectrum

The remarkably low volatility of Iopamidol, due to its high molecular weight and to the presence of several polar functional groups, complicates its mass spectral characterization.

Partial information has been gained by chemical derivatization procedures combined with different ionization methods and conclusive mass spectral characterization of Iopamidol was obtained when the fast atom bombardment (FAB) technique was employed (9).

The FAB mass spectrum of Iopamidol in glycerol is shown in Fig. 6.

As can be observed the base peak corresponds to the protonated molecular ion (m/z 778).

The fragmentation pattern of this [M+H]<sup>+</sup> species is shown in Scheme 1.

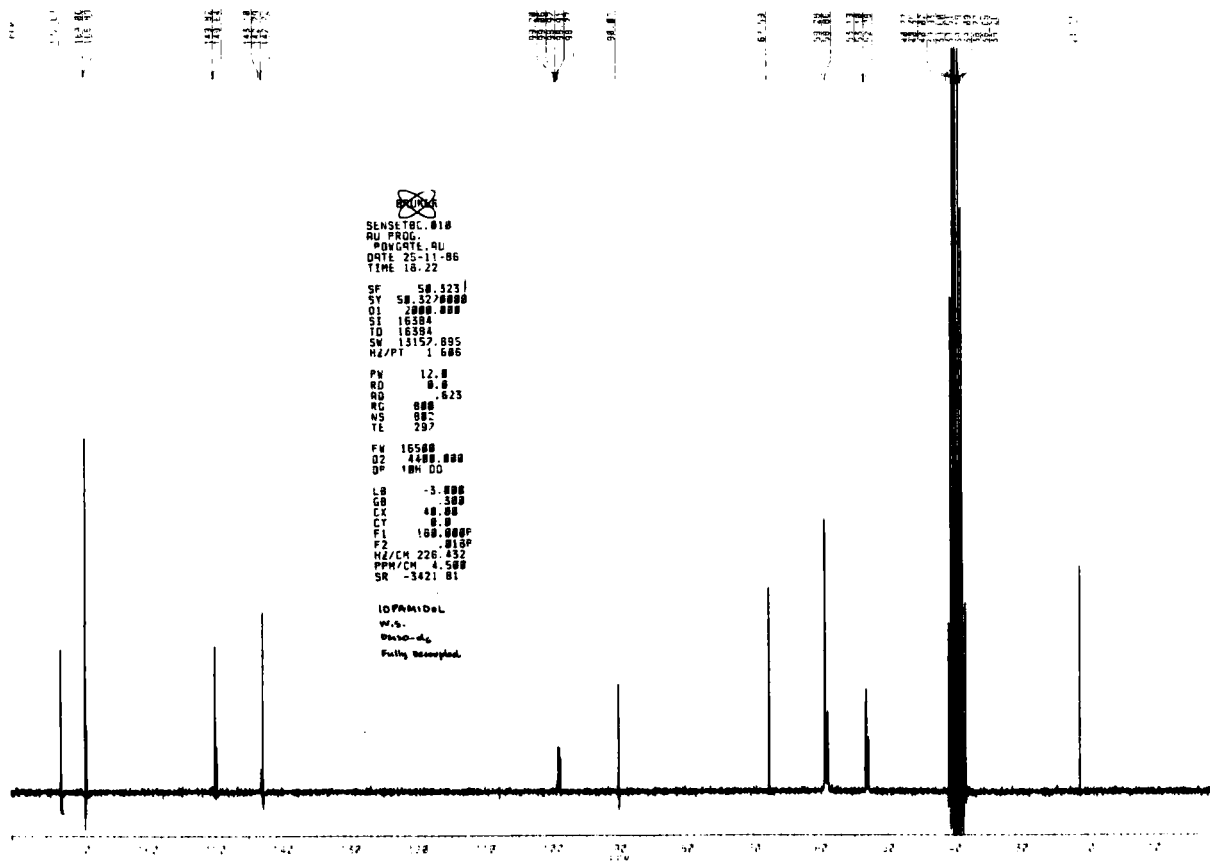


Fig. 5 -  $^{13}\text{C}$ -NMR spectrum of Iopamidol in DMSO



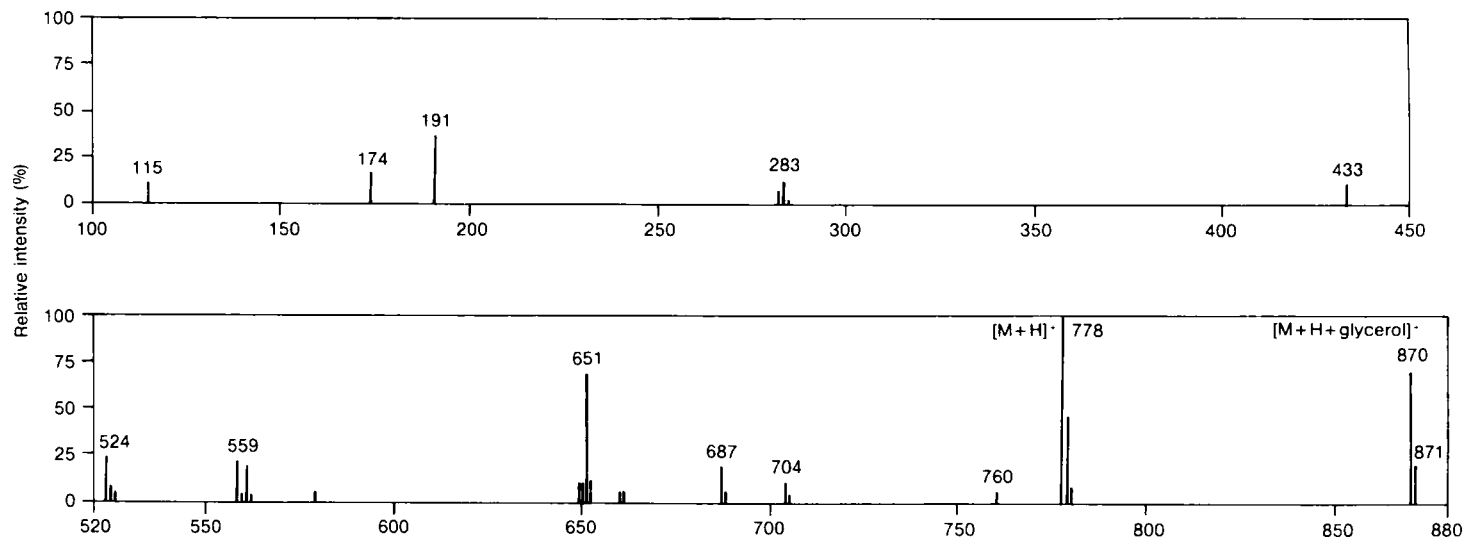


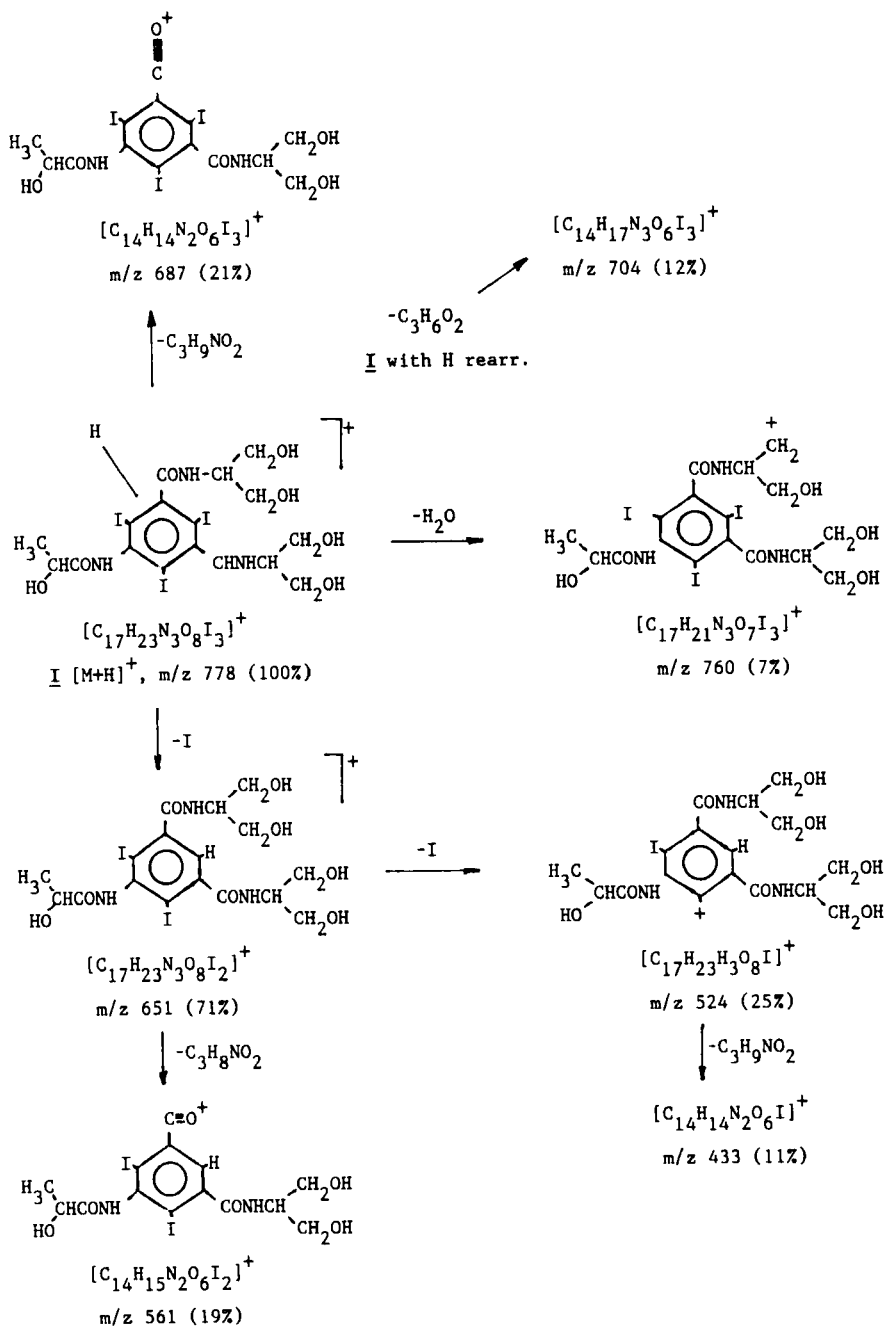
Fig. 6 - FAB MASS spectrum of Iopamidol

Table 5

 $^{13}\text{C}$ -NMR data (50 MHz) in DMSO-d<sub>6</sub>

Chemical shift $\delta_{\text{C}}$ (ppm, TMS)	Assignment (s)
172.67	$\phi\text{NHCO}$
169.06	$\phi\text{CONH}$
168.89	
149.82	Aromatics C-3, C-5
149.64	
143.10	Aromatic C-1(N)
142.79	
142.72	
99.20	Aromatics C-2, C-6
99.06	
98.97	
98.91	
98.77	
90.07	Aromatic C-4
67.59	CH, lactoyl
59.28	$\text{CH}_2$ , serinol
58.86	
53.19	CH, serinol
53.10	
52.78	
22.11	$\text{CH}_3$

**SCHEME 1**  
FRAGMENTATION PATTERN OF IOPAMIDOL



## 3.2 Solid state properties

### 3.2.1 Crystal morphology

Iopamidol exists in three different crystal forms, corresponding to the anhydrous, the monohydrate and the pentahydrate, each characterized by a distinct IR Spectrum (see 3.1.2), X-ray powder diffraction pattern (see 3.2.2) and by distinct both enthalpimetric (see 3.2.4) and gravimetric ( see 3.2.5) thermograms.

Crystals of the three forms, which were induced to grow with great difficulty from aqueous solutions, were subjected to structural X-ray analysis using a Philips PW 1100 diffractometer ( Mo K  $\alpha$  = 0.71069 Å; scan speed = 0.06°/s) for the anhydrous form and a CAD-4 diffractometer for the mono and the pentahydrate forms.

Crystal cell parameters of each of the three forms are reported in Table 6.

Table 6  
Crystal data of Iopamidol

Parameter	Anhydrous (10)	Monohydrate (11)	Pentahydrate (12)
a (Å)	12.478	15.591	14.147
b (Å)	11.233	13.611	12.484
c (Å)	9.241	12.608	15.963
$\alpha$ (degr.)	104.49	81.41	90.00
$\beta$ (degr.)	92.63	62.25	90.69
$\gamma$ (degr.)	108.63	87.10	90.00
V (Å <sup>3</sup> )	1117	2426	2819
Dx(g/cm <sup>3</sup> )	2.19	2.18	2.04
Z	2	4	4
Space group	P <sub>1</sub>	P <sub>1</sub>	P <sub>21</sub>

Structural analysis of the anhydrous and of the pentahydrate forms further confirmed what was observed by NMR spectroscopy (see 3.1.3) as regards the ENDO conformation of the CO-N bond (anhydrous and penta- hydrate) and the ANTI E,E (pentahydrate) and SYN E,E (anhydrous) conformations of the isophthalic bonds  $\emptyset$ -CONHR.

### 3.2.2 X-Ray Powder Diffraction

X-Ray powder diffraction patterns of the three forms of Iopamidol were obtained with a Philips PW 1710 diffractometer in the  $2\theta$  range between 3 and  $50^\circ$  using a Cu-Ni radiation (40 KV; 40 mA) and a  $1^\circ/\text{min}$  scanning rate (13). Data obtained on samples crystallized from water are reported in Tables 7, 8 and 9 and clearly demonstrate that the three forms yield three distinct diffractograms.

Table 7  
X-ray Powder Diffraction Pattern of anhydrous Iopamidol

D (Å)	I%rel.	D (Å)	I% rel.
11.73	100	2.99	22
10.24	40	2.94	22
9.49	34	2.90	39
8.88	24	2.88	27
7.89	49	2.85	41
7.61	27	2.79	20
6.66	33	2.74	14
6.04	35	2.73	14
5.86	19	2.69	46
5.10	58	2.62	16
5.05	74	2.58	32
4.73	72	2.54	13
4.56	97	2.43	12
4.46	17	2.41	12
4.27	42	2.37	18
4.23	31	2.34	24
4.18	33	2.27	16
4.15	22	2.25	12
3.97	32	2.23	19
3.94	37	2.21	21
3.90	66	2.19	13
3.80	31	2.17	17
3.77	21	2.11	15
3.71	37	2.09	16
3.61	33	2.07	14
3.58	44	1.99	13
3.52	37	1.96	14
3.42	17	1.88	16
3.40	14	1.85	16
3.29	42		
3.15	16		
3.08	51		

Table 8

X-Ray Powder Diffraction Pattern of monohydrate Iopamidol

D (Å)	I%rel.	D (Å)	I%rel.
13.48	14	3.11	14
11.38	50	3.07	8
9.68	100	3.04	10
8.19	14	3.01	7
7.15	12	2.97	7
6.88	9	2.94	9
6.32	41	2.88	11
6.16	12	2.86	15
6.05	11	2.84	9
5.72	46	2.80	10
5.53	13	2.76	20
5.49	19	2.73	20
5.28	12	2.61	19
5.23	12	2.54	6
5.03	11	2.50	10
4.86	33	2.46	7
4.69	8	2.44	6
4.60	8	2.37	6
4.56	12	2.34	5
4.49	12	2.32	5
4.26	14	2.27	7
4.20	12	2.26	6
4.10	10	2.20	6
3.99	20	2.13	8
3.87	6	2.11	9
3.80	17	2.09	8
3.70	11	2.05	15
3.67	9	2.00	20
3.57	17	1.95	5
3.52	10	1.86	7
3.44	15	1.70	5
3.41	13	1.65	6
3.32	11	1.62	9
3.27	16	1.62	6
3.17	8		

Table 9

X-Ray Powder Diffraction Pattern of pentahydrate Iopamidol

D (Å)	I%rel.	D (Å)	I%rel.
14.12	74	3.16	15
10.62	29	3.08	72
9.79	51	3.03	13
9.33	51	2.90	25
8.35	29	2.87	36
7.78	36	2.85	57
7.33	40	2.82	100
7.06	30	2.79	25
6.71	83	2.77	33
6.59	60	2.71	36
6.23	36	2.69	20
6.14	34	2.65	25
5.82	21	2.60	30
5.70	20	2.57	24
5.53	45	2.54	18
5.14	38	2.51	15
4.98	57	2.45	27
4.89	24	2.42	22
4.79	44	2.40	15
4.62	35	2.31	17
4.58	20	2.27	12
4.46	30	2.25	19
4.38	67	2.22	25
4.29	22	2.18	15
4.18	16	2.14	26
4.03	44	2.09	23
3.98	35	2.06	20
3.89	20	1.99	25
3.83	29	1.95	21
3.75	33	1.93	21
3.67	86	1.92	18
3.59	18	1.90	24
3.57	17	1.88	18
3.44	13	1.84	12
3.38	59	1.81	16
3.35	28	1.74	13
3.27	39	1.71	13
3.22	34	1.69	16
3.19	30	1.56	14

### 3.2.3 Melting Range and Eutectic Temperature

Melting point determination of anhydrous Iopamidol, alone or in mixture with suitable substances, was made with a Kofler microscope operating as described by Roth (14). The product shows no change up to a temperature of 290°; then the crystal becomes black and a slow decomposition begins.

In mixtures with benzil, acetanilide, phenacetine and benzanilide, the compound does not change the m.p. of the mixed substances while it lowers the m.p. of salophen (186°;  $\Delta T = 2.6^\circ$ ) and cyanoguanidine (180°;  $\Delta T = -28.7^\circ$ ).

### 3.2.4 Differential thermal analysis

Differential thermal analysis was carried out using a Mettler TA 3000 calorimeter in the range 30-300°C with a heating rate of 10°/min and a purge (air) of about 25 ml/min (15). Figure 7 shows the thermograms of the anhydrous, monohydrate and pentahydrate forms of Iopamidol which exhibit three clearly distinct patterns characterized as follows :

- **anhydrous form** : monotonous curve with a wide (140-220°) endothermic transition of small intensity
- **monohydrate form** : three endothermic transitions at 115° ( $\Delta H \approx 55$  J/g), 250° and 265° the latter two not resolved, with  $\Delta H$  of about 40 and 67 J/g. The first transition is associated with the loss of one molecule of water.
- **pentahydrate form** : four endothermic transitions at 87° ( $\Delta H \approx 215$  J/g), 117° ( $\Delta H \approx 55$  J/g), 183° ( $\Delta H \approx 10$  J/g) and 255° ( $\Delta H \approx 30$  J/g). The first two transitions are ascribable to loss of 4 and 1 moles of water, respectively.

Above 300° all the forms undergo decomposition with release of iodine.



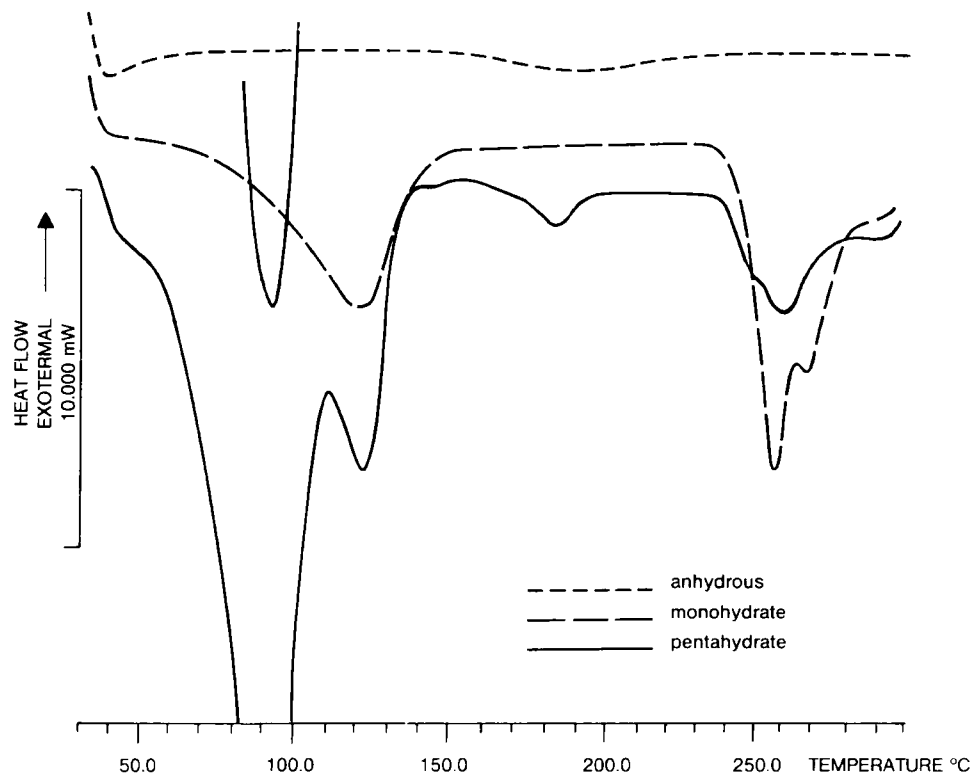


Fig. 7 - DTA of various forms of Iopamidol

### 3.2.5 Thermogravimetric analysis

Thermogravimetric analysis of the anhydrous, the monohydrate and the pentahydrate forms of Iopamidol were carried out using a Mettler apparatus consisting of a thermobalance TG50, a TA processor TC10A and a printer, in the temperature range 30-200° and with a heating rate of 10°/min (15). The results showed that the monohydrate form loses 2.0% (0.9 moles of water) of its weight around 108°, the pentahydrate 8.1% (4 moles of water) at 80° and another 2.3% (1 mole of water) at about 104°, while the anhydrous form remains unchanged. The behaviour observed is consistent with what deduced from differential thermal analysis.

## 3.3 Solution properties

### 3.3.1 Optical rotation

Optical rotation of an aqueous solution of Iopamidol was studied by Felder (16), who reported that optical rotation of a 10% aqueous solution is quite low:  $[\alpha]_{20^\circ, 589\text{nm}} = -3.20^\circ \pm 0.01^\circ$  and scarcely influenced by temperature :  $[\alpha]_{T, 589\text{ nm}} = -2.762^\circ \pm 0.022 T \pm 0.010^\circ$  ( $20^\circ < T < 40^\circ$ ). In the region  $589 \div 365\text{ nm}$  it resulted that the optical rotatory dispersion curve could be expressed by the single equation of Drude :  $[\alpha]_{20^\circ, \lambda} = -0.6871^\circ/\lambda^2 - 0.056 \pm 0.024$  ( $\lambda = \mu\text{m}$ ). Moreover, the Author described a method for the determination of optical purity of the product by enzymatic reaction with L- and D-lactic dehydrogenase following reductive dehalogenation of Iopamidol and subsequent alkaline hydrolysis, and also the possibility of increasing considerably the specific rotatory power of Iopamidol by complexation with Cu (II) ions in presence of alkali. A value of  $[\alpha]_{20^\circ, 436\text{nm}} = 142.2^\circ \pm 0.11^\circ$  ( $c=2.5\%$ ; water) was found for the complex ML2 using a product with a purity corresponding to  $98.9 \pm 0.5\%$  of L form and  $1.62 \pm 0.19\%$  of D form.

### 3.3.2 Solubility in water

The question of the water-solubility of Iopamidol cannot be univocally answered. In fact, in the same range of temperatures, different solubility curves exist according to the re-

spective nature of the crystalline phases at the equilibrium (Fig.8). In the absence of any crystalline phase at the start, even solutions of extremely high concentrations ( $>>400$  mgI/ml), while being proportionally more viscous up to the limit of vitreous consistency, can last indefinitely without ever forming a new phase.

Three crystalline phases, different as to their lattice spacings, molecular conformations, and degrees of hydration, have been isolated and characterized with various techniques ( see 3.1.2, 3.2.1, 3.2.2, 3.2.4, 3.2.5) and their respective solubility curves are depicted in Fig.8.

### 3.3.2 Ionization constant

The acid dissociation constant of Iopamidol was determined by potentiometric titration in water (17). The pKa value is 10.70 at 25°. Accordingly, the pH of an unbuffered molar aqueous solution is 5.3 and the degree of ionization at pH = 7.0 is only 0.02%.

### 3.3.4 Partition coefficient

Partition coefficients were determined at 20° in n-octanol/0.01M phosphate buffer pH 7.4 and in n-butanol/0.01M phosphate buffer pH 7.4 according to Leo (18).

The values found are given in Table 10.

Table 10  
Partition coefficients at pH 7.4 and 20°

Organic phase	P $\pm$ sd
n-Octanol	0.0025 $\pm$ 0.0001
n-Butanol	0.094 $\pm$ 0.005

### 3.3.5 Density 20/20°

Densities of aqueous solutions of Iopamidol were measured at 20° using a 1 ml Pregl pipette (14).

In the concentration range  $c = 10\div 80$  g% ml the

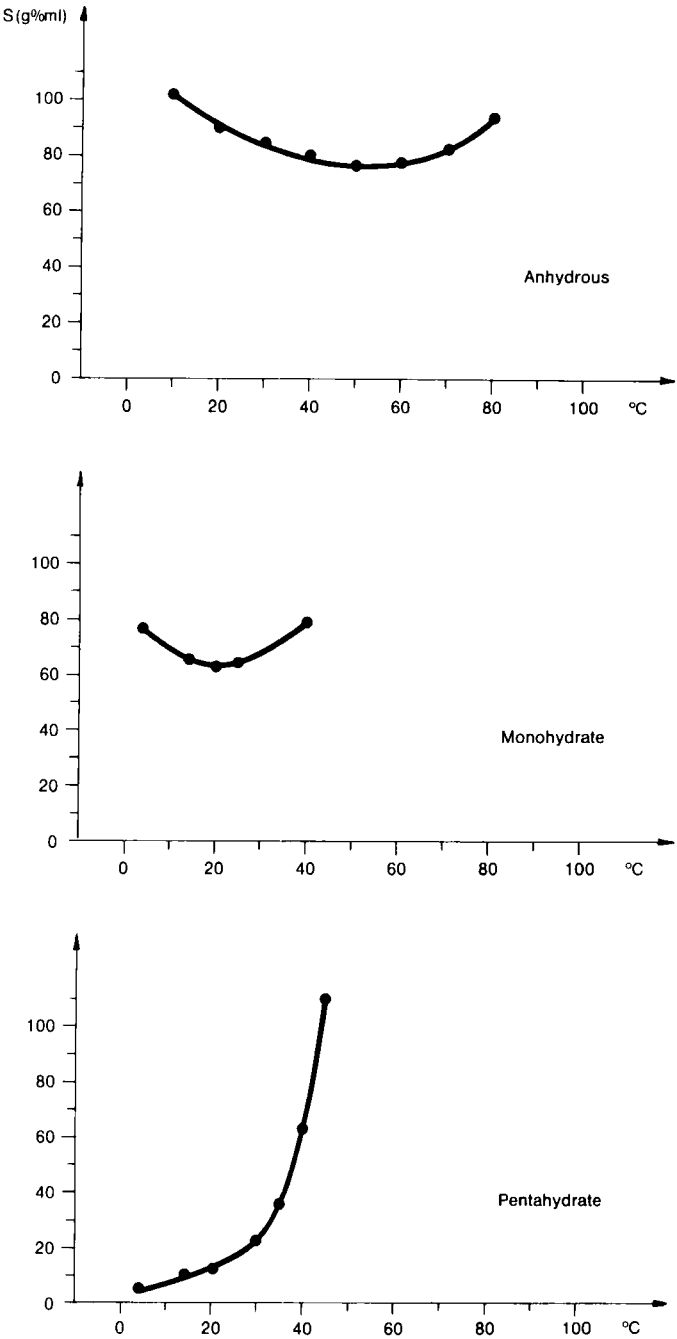


Fig. 8 - Solubility profile of various forms of Iopamidol in water

following relationship was found :

$$d_{20/20} = 0.99669 + 0.0055033 \cdot c \pm 0.00035 ; \\ r=0.999997.$$

### 3.3.6 Refractive index

The refractive index of a series of aqueous solutions of Iopamidol was determined at 20° with an Abbe-ATAGO refractometer.

The following relationship was found in the  $c = 10 \div 80\text{g\% ml}$  concentration range :  $n_{20^\circ}$ ,  $D = 1.33240 + 0.0016386 \cdot c \pm 0.00055$ ;  $r=0.999984$ .

### 3.3.7 Viscosity

Viscosity measurements of a series of aqueous solutions of Iopamidol were carried out at 20° and at 37° using a Haake Rotovisco RV-100 Viscosimeter. Before testing, aqueous solutions were filtered through a 0.45  $\mu\text{m}$  Millipore membrane and sterilized at 120° for 30 minutes.

Table 11 lists the mean values found (19).

Table 11

Iopamidol viscosity data

Concentration (mgI/ml)	$\eta$ Viscosity. ( $\text{mP.s}^{-1}$ )	
	20°	37°
100	1.6	1.1
150	2.3	1.5
200	3.3	2.1
250	5.0	3.1
300	8.3	4.4
370	18.4	9.0

The relationship between viscosity and temperature can be expressed by the following exponential equations :

$$\eta^{20} = 0.593 \cdot e^{0.009 \cdot c} ; \quad r = 0.989$$

$$\eta^{37} = 0.475 \cdot e^{0.008 \cdot c} ; \quad r = 0.991$$

### 3.3.8 Osmotic properties

Osmolality, osmotic pressures and osmotic coefficients of a series of aqueous solutions of Iopamidol, previously sterilized at 120° for 30 min, were determined both by cryoscopy (Advanced Mod 3 W II, Advanced Instruments) and by vapor pressure measurements (Knauer, vapor pressure osmometer); in the latter case measurements were carried out at 37° after calibrating the instrument with aqueous mannitol solutions (20). Values are reported in Table 12 and 13.

Table 12  
Iopamidol Osmometric data by cryoscopy

Concentrations				Values found (23)		
mol/l	mol/kg	g%ml	mgI/ml	$\psi_{vm}$ (osmol/kg)	$\pi$ (atm)	$\psi$
0.263	0.291	20.4	100	0.236	6.01	0.81
0.394	0.459	30.6	150	0.346	8.81	0.75
0.525	0.646	40.8	200	0.465	11.8	0.72
0.657	0.857	51.1	250	0.594	15.4	0.69
0.788	1.092	61.2	300	0.740	18.8	0.68
0.972	1.479	75.5	370	(*)	-	-

(\*) does not freeze

Table 13  
Vapor pressure osmometric data of Iopamidol at 37°

Concentrations				Values found		
mol/L	mol/Kg	g%ml	mgI/ml	$\psi_{vm}$ (*) (osmol/Kg)	$\pi$ (**) (atm)	$\psi$ (***)
0.263	0.291	20.4	100	0.224	5.70	0.77
0.394	0.459	30.6	150	0.318	8.09	0.69
0.525	0.646	40.8	200	0.416	10.6	0.64
0.657	0.857	51.1	250	0.513	13.1	0.60
0.788	1.092	61.2	300	0.620	15.8	0.57
0.972	1.479	75.5	370	0.799	20.3	0.54

(\*) Osmolality =  $\ln a/v \cdot d_0$  (21,22)

(\*\*) Osmotic pressure =  $\psi_{vm}RT$

(\*\*\*) Osmotic coefficient

The explanation of the fact that the cryoscopic method yields higher values, lies in the non-ideal behaviour of Iopamidol solutions. Studies carried out on 0.1 : 1.5 mol/kg Iopamidol solutions using the classic cryoscopic method for freezing point determination (24), confirmed this tendency and also showed that the composition of the solid phase which separates upon freezing, e.g. from a solution at 370 mgI/ml, is virtually unchanged with respect to the starting solution. A similar behaviour was previously observed by Børdalen (21) in his study on the osmotic properties of aqueous solutions of conventional contrast media.

For these reasons we consider that, for this class of compounds, data obtained with the vapor pressure method are more reliable.

### 3.3.9 Surface Tension

The surface tension at 20° of a series of Iopamidol aqueous solutions was determined using an interfacial tensiometer according to Lecomte Du Nouy (Krüss).

Values recorded immediately after preparation of the solutions (time zero) and after an equilibrium period of 16 hours are reported in Table 14.

Table 14

Surface tension at 20°

Iopamidol concentration			Surface tension (dyne/cm)	
g%ml	mgI/ml	mol/L	Start	Equilibrium
10.2	50	0.131	68.7	55.4
20.4	100	0.263	65.3	52.5
40.8	200	0.525	60.7	51.1
61.2 <sub>5</sub>	300	0.788	59.0	50.8
81.6 <sub>5</sub>	400	1.051	58.3	50.6

### 3.9.10 Critical Micelle Concentration (c.m.c.)

Critical micelle concentration value of Iopamidol

in water was determined according to the method of "spectral variation" described by Thoma (25) using Eosin Yellowish, Rodamine 6G and Pinacyanol Chloride. The results are listed in Table 15.

Table 15

Critical micelle concentration

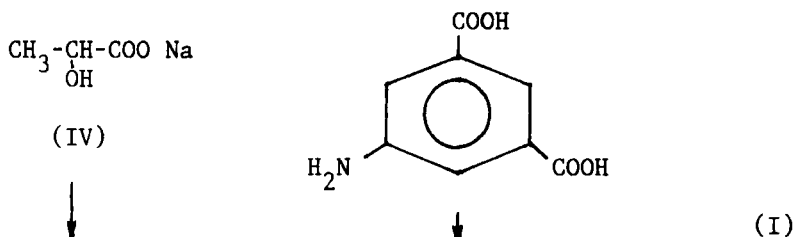
Dyestuff	Analytical (nm)	c.m.c.	
		g% ml	mol/L
Eosine Yellowish	532.0	3.8	$4.9 \cdot 10^{-2}$
Rodamine 6G	543.5	3.8	$5.0 \cdot 10^{-2}$
Pinacyanol	619.0	2.8	$3.6 \cdot 10^{-2}$

#### 4. SYNTHESIS

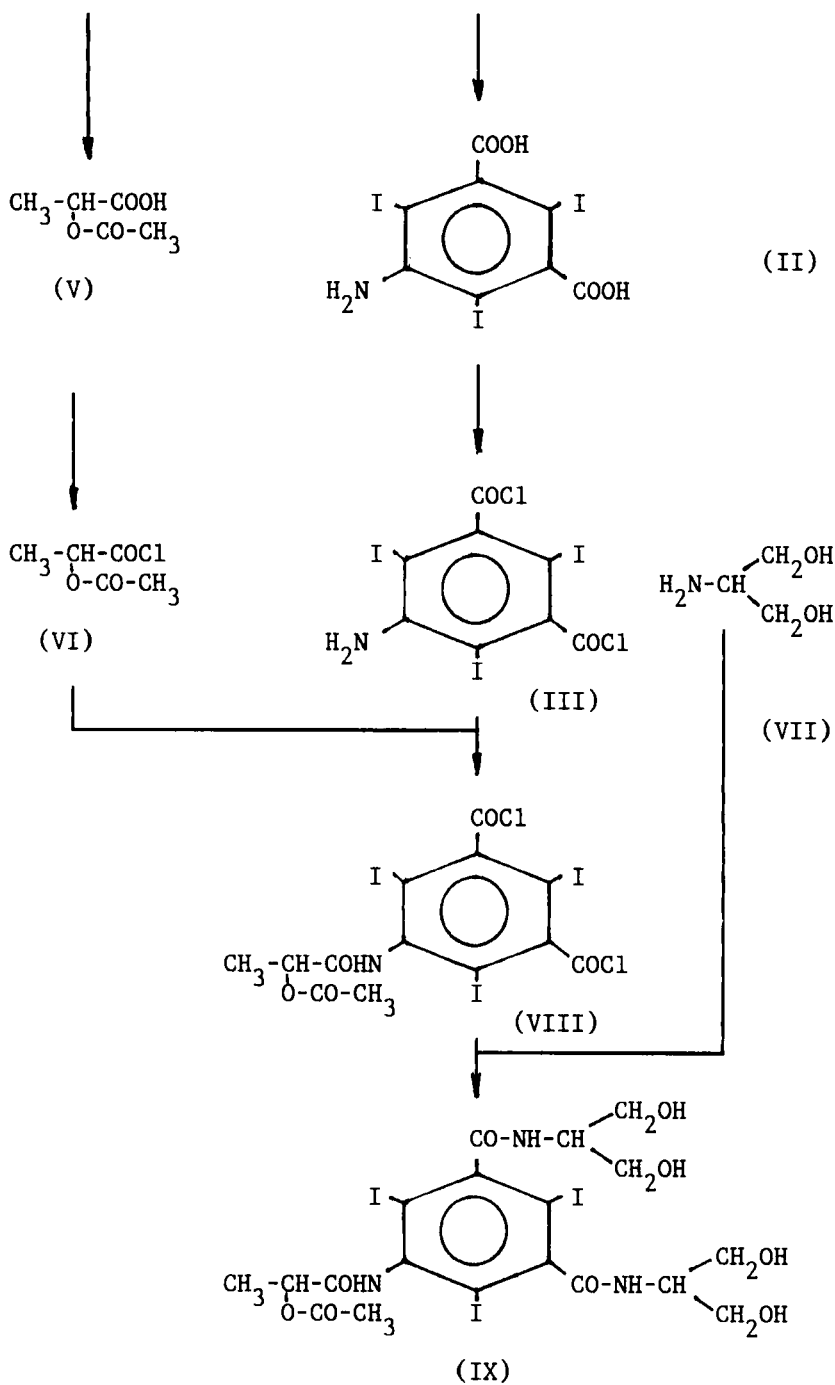
In Iopamidol synthesis, which was developed by Felder (3) and is outlined in Scheme 2, the starting product aminoisophtalic acid (I) is first iodinated at the 2,4 and 6 positions (II) and then its dichloride (III) is subjected to N-acylation with the chloride of the O-acetyl-L-lactic acid (VI), to condensation with 2-aminopropan-1,3-diol (VII) (Serinol) and finally transformed into the final product (X).

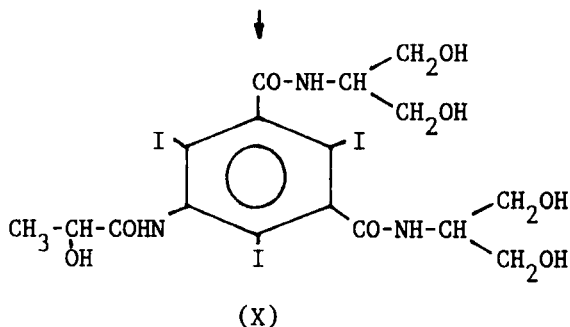
During development of the synthesis on the industrial scale, each single step was further studied as to its kinetics and was optimized in order to reduce to a minimum the possibility of by-products formation and of racemization.

**SCHEME 2**  
Iopamidol Synthesis









## 5. STABILITY AND DEGRADATION

Under normal storage conditions Iopamidol is stable at room temperature.

The main potential degradation routes of the product in its injectable formulation were reported by Felder (26).

## 6. METHODS OF ANALYSIS

### 6.1 Elemental analysis

Element	%calc.	%found
C	26.27	26.40
H	2.85	2.94
N	5.41	5.66
I	49.00	48.98
O	16.47	16.22

### 6.2 Identification tests

The identification of Iopamidol can be carried out according to the following methods :

- a) heating on a flame about 50 mg of product in a tube : violet iodine vapors are evolved.
- b) the UV spectrum of a solution containing about 20 mg/l of product in pH=9 phosphate buffer, shows an absorption maximum at 242 nm ( E 1%, 1 cm = 380)..

- c) the IR spectrum of the product in KBr pellet shows characteristic absorption bands at : 3380, 3240, 2940, 2880, 1630, 1530, 1350, 1245, 1045, 970 and 890  $\text{cm}^{-1}$ .

### 6.3 Organically bound iodine

Organically bound iodine is determined potentiometrically in acidic solution by titration with 0.1N  $\text{AgNO}_3$  after reductive dehalogenation with 2N NaOH (6) or with  $\text{NaBH}_4$  according to the method described by Egli (27).

### 6.4 Chromatography

#### 6.4.1 Thin layer chromatography

The identification and separation of Iopamidol from its byproducts by thin layer chromatography is summarized in Table 16.

Table 16  
TLC data for Iopamidol (28)

Solvent System	Rf <sub>a</sub>	Rf <sub>b</sub>
I	0.18	0.24
II	0.39	0.30
III	0.58	0.52
IV	0.26	0.30
V	0.28	0.21
VI	0.28	0.30
VII	0.43	0.47
VIII	0.31	0.30
IX	0.36	0.36
X	0.62	0.72
XI	0.45	0.45
XII	0.32	0.14

#### Support

- a) Silica gel 60 F<sub>254</sub>, precoated plates, Merck  
b) Cellulose F, precoated plates, Merck

#### Solvent System

I	$\text{CHCl}_3$ : MeOH : 25% $\text{NH}_3$	6:3:1 (v/v)	
II	$\text{CHCl}_3$ : MeOH : 25% $\text{NH}_3$	6:5:1	"
III	$\text{CHCl}_3$ : MeOH : 25% $\text{NH}_3$	5:4:2	"
IV	$\text{CH}_3\text{COOC}_2\text{H}_5$ : EtOH:25% $\text{NH}_3$	15:7:6	"
V	$\text{CH}_3\text{COOC}_2\text{H}_5$ : iso-PrOH:25% $\text{NH}_3$	2:2:1	"
VI	iso-PrOH : 25% $\text{NH}_3$	4:1	"
VII	$\text{CH}_3\text{COCH}_3$ : iso-PrOH :25% $\text{NH}_3$	2:2:1	"
VIII	n-ButOH : MeOH : 25% $\text{NH}_3$	4:1:1	"
IX	sec-ButOH : iso-PrOH: 25% $\text{NH}_3$	5:2:3	"
X	$\text{C}_2\text{H}_5\text{COCH}_3$ : n-PrOH: EtOH : 25% $\text{NH}_3$	10:1:2:7	"
XI	$\text{C}_2\text{H}_5\text{COCH}_3$ : $\text{CH}_3\text{COOH}$ : $\text{H}_2\text{O}$	15:3:5	"
XII	$\text{CH}_3\text{COOC}_2\text{H}_5$ : $\text{CH}_3\text{COOH}$ : $\text{H}_2\text{O}$	5:2:1	"

- Detection

- . Ultraviolet (254 nm)
- . 1% aqueous starch and subsequent exposure to UV light (254 nm) to give brown spots.

The best results in terms of separation of Iopamidol from its principal potential impurities were achieved using solvent system I with support a.

#### 6.4.2 High Pressure Liquid Chromatography

The following HPLC method was developed for qualitative and quantitative determination of Iopamidol and of its by-products (29) :

- Apparatus : HPLChromatograph H.P.1084B  
with variable wavelength  
detector set at 240 nm
- Column : Lichrosorb RP18-5 $\mu\text{m}$  : 4x250 mm
- Injection : 20  $\mu\text{l}$

- Eluant A : water
- Eluant B : methanol 25% in water (v/v)
- Flow rate : 1.5 ml.min<sup>-1</sup>
- Gradient profile :

min	%B
0	7.5
6	7.5
18	35.0
30	92.0
34	92.0
37	7.5
42	7.5

column recondi-  
tioning
- Column temperature : 35°
- Retention time of Iopamidol : ~7.5 min

## 6.5 Analysis of impurities

A certain number of the most probable potential impurities arising from Iopamidol synthesis were characterized both chromatographically (TLC, HPLC) and structurally using spectroscopic methods, i.e. UV, IR, <sup>1</sup>H-NMR, FAB/MS (30).

### 6.5.1 Free aromatic amine

A manual method (6), based on the classic colorimetric reaction according to Bratton and Marshall, was set up for the determination of free aromatic amine; the procedure was automated for routine analysis both of the bulk product and of its injectable formulations (31).

### 6.5.2 Free iodine and free halides

Free iodine is detected by extraction with toluene of an acidic (H<sub>2</sub>SO<sub>4</sub>) aqueous solution of Iopamidol (2g/30 ml). Toluene must remain colorless (6). Free halides are determined by addition of AgNO<sub>3</sub> to an acidic aqueous solution of Iopamidol and comparison of its turbidity with that of solutions containing a known amount of chlorides (6).

## 7. METABOLISM AND PHARMACOKINETICS

### 7.1 Metabolism

The metabolic fate of Iopamidol was studied in rabbits and dogs after intravenous and intrathecal administration (32, 33, 34, 35).

Both species excrete the compound unchanged, as demonstrated by thin layer chromatography of the urine and bile and by isolation from urine. Human studies (34, 36, 37) have shown that Iopamidol does not undergo any significant biotransformation or deiodination after intravenous as well as after intrathecal administration.

### 7.2 Pharmacokinetics

Several authors (32, 34, 35, 38, 39, 40, 41, 42) studied the pharmacokinetics of Iopamidol in various animal species both after intravenous and after intrathecal dosing.

When administered intravenously, the compound distributes to a volume equivalent to the extracellular fluid and is filtered almost exclusively by the kidneys. Also after intrathecal administration Iopamidol is eliminated rapidly through the kidneys. The results of human studies (34, 36, 37, 43, 44, 45, 46, 47) are in general agreement with the data obtained in experimental animals. After intravenous injection the pharmacokinetics of Iopamidol is best described by an open linear, two-compartment model. The average plasma elimination half-life is about 2 hours. The volume of distribution is approximately equal to the extracellular fluid volume. Iopamidol is excreted predominantly through the renal route. Fecal elimination averages 1% or less of the dose, indicating minimal biliary excretion.

The distribution and elimination kinetics of Iopamidol after intrathecal injection was assessed by densitometric CT readings and by iodine assay of blood and urine (43).

CT readings were maximal at 1hr in the lumbosacral subarachnoid space and at 6hr in the cervical region.

Peak plasma levels were observed at 2.9 hr and were no longer detectable at 48 hr. The 48 hr urinary recovery averaged  $66 \pm 8\%$ .

After oral administration to the rat, Iopamidol was

eliminated within 48 hr almost totally by the fecal route and only negligible amounts were found in urine (42).

### 7.3 Protein Binding

No binding of Iopamidol to plasma proteins and CSF proteins of dog and rabbit was observed (32). Human serum protein binding averaged less than 1% at 1hr postinjection (36).

## 8. DETERMINATION OF IOPAMIDOL IN BODY FLUIDS AND TISSUES

Most methods for assay of contrast media are based on the assumption that the amount of contrast medium in the sample is proportional to its iodine content.

This assumption is valid also for assay of Iopamidol in body fluids and tissues, since the injectable solutions contain only negligible amounts of  $I^-$  and no appreciable deiodination occurs in vivo (37).

A survey on methods for assay of iodinated contrast media has recently been published (49).

The following methods have been reported for assay of Iopamidol :

### 8.1 Colorimetry

A fully automated colorimetric method, originally developed for the determination of protein bound iodine (PBI) (50, 51), has been applied by several authors (32, 34, 36, 46, 60) for assay of Iopamidol in plasma, urine and CSF.

### 8.2 X-Ray Fluorescence

X-ray fluorescence analysis, developed for determination of iodine in vitro (48,52) and in vivo (52, 53, 61) was also utilized in several pharmacokinetic studies with Iopamidol (36, 43, 44, 54, 55, 62, 63, 64, 65).

Experimental details, precision and accuracy of X-ray fluorescence analysis of Iopamidol in body fluids and tissues by excitation of the  $L\alpha$  line of iodine are reported by V.Lorusso et al. (55).

### 8.3 Neutron activation

The high sensitivity of neutron activation analysis, which allows the measurement of iodine concentrations as low as 0.02 ppm, was used by O. Muratore et al.(40) for the study of iodine levels in different tissues of rats up to 62 days after injection of Iopamidol.

### 8.4 Radioactive labeling

Both  $^{125}\text{I}$ - and  $^{14}\text{C}$ -labeled Iopamidol have been employed for study of its pharmacokinetics and tissue distribution [ Franchini  $^{125}\text{I}$  (45), Kivisaari  $^{125}\text{I}$  (56), Rosenbaum  $^{14}\text{C}$  (39), Mc Kinstry  $^{14}\text{C}$  (36)].

### 8.5 CT-Densitometry

Although less sensitive than the above mentioned methods, CT-densitometry, i.e. X-ray absorption measurements during computerized tomography, has been successfully utilized for evaluation of Iopamidol concentrations in vivo and in vitro (38, 57, 58).

### 8.6 High performance liquid chromatography [HPLC]

A specific and precise HPLC method for assay of Iopamidol in urine, plasma and CSF, applicable in the range 5-1000 mcg/ml for urine and 0.5-5000 mcg/ml for plasma and CSF has been reported by E.Felder et al. (59).

HPLC was also employed in the studies of D.Pitrè et al. (37) and D.Mc. Kinstry et al. (36).



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# Ivermectin

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## IVERMECTIN

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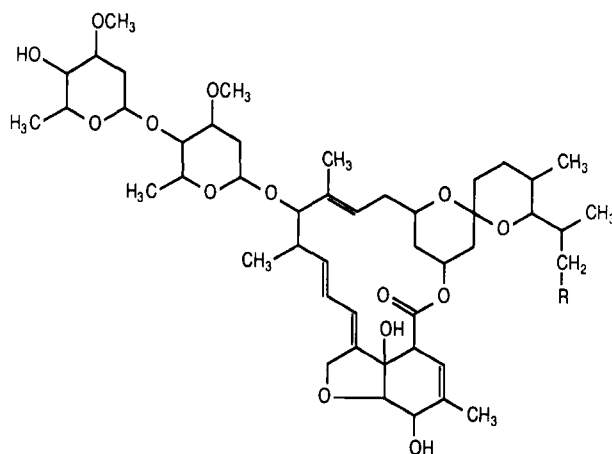
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## I. INTRODUCTION

Ivermectin is an antiparasitic agent derived from naturally-occurring fermentation products which exhibits a broad spectrum of activity (1) with a potency far exceeding those of other anthelmintics. Numerous reports describing the efficacy of this drug against both internal and external parasites in a wide variety of hosts have appeared in the literature. Although the major uses of ivermectin are in veterinary applications to cattle, sheep, swine, horses and dogs, it is also an effective drug for treatment of onchocerciasis (river blindness) in man (2,3). One comprehensive publication includes descriptions of the efficacy, chemistry, microbiology, isolation, structure determination, and metabolic disposition of ivermectin (4).

Ivermectin, a semi-synthetic derivative of the avermectins, is composed of a mixture of two homologous compounds (5) each of which is prepared from the avermectin family of fermentation products by selective reduction of an olefin on two of these naturally-occurring species. These parent avermectins are disaccharide ( $\delta$ -L-oleandrosyl- $\delta$ -L-oleandroside) derivatives of pentacyclic 16-membered lactones (6). (See SYNTHESIS section below for the structures of the general class of the avermectin family of precursor compounds.)

The structure of ivermectin (I) includes a dihydroxycyclohexene ring fused to a tetrahydrofuran moiety. Compound a ("H<sub>2</sub>B<sub>1a</sub>") is 5-O-demethyl-22,23-dihydroavermectin A<sub>1a</sub> and homolog b ("H<sub>2</sub>B<sub>1b</sub>") is 5-O-demethyl-25-de(1-methylpropyl)-22,23-dihydro-25-(1-methyl-ethyl)avermectin A<sub>1a</sub>.



Component	R
a	CH <sub>3</sub>
b	H

I

## Structure of Ivermectin (I)

The empirical formulas and molecular weights of the a and b homologs of ivermectin are C<sub>48</sub>H<sub>74</sub>O<sub>14</sub>, MW = 875.10 and C<sub>47</sub>H<sub>72</sub>O<sub>14</sub>, MW = 861.07, respectively. Because ivermectin is defined as a mixture consisting of ≥80% of compound a and <20% homolog b, the molecular weight of this drug will range from 872.21 to 875.10.

Ivermectin is an off-white nonhygroscopic crystalline powder. It has 19 asymmetric centers and is optically active,  $[\alpha]_D +71.5 \pm 3^\circ$  (c = 0.755 in chloroform).

## II. SPECTRAL PROPERTIES

### A. Nuclear Magnetic Resonance

#### 1. Carbon-13

Carbon-13 NMR spectra were recorded on a 10% w/v solution of ivermectin in  $\text{CDCl}_3$  with TMS as reference. A Bruker WM250 spectrometer operating at 62.9 Mhz was used with broadband proton decoupling at 250 Mhz (7). Proton population assignments on various carbons were made using the Attached Proton Test (APT) experiment: Patt, S. L.; Shoolery, J. N. J. Magn. Reson. 1982, 46, 535-539. This spectrum is shown in Figure 1 and the chemical shift assignments are listed in Table I.

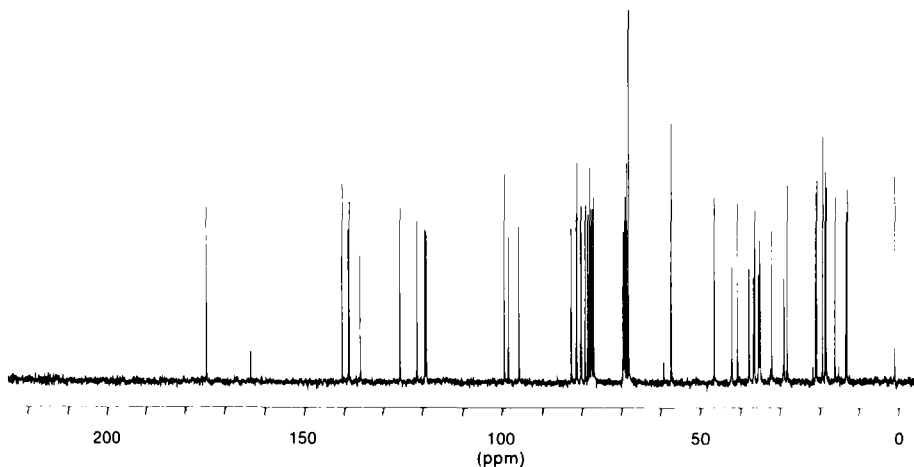


Fig. 1. Carbon-13 NMR Spectrum of Ivermectin. 10% w/v Solution in  $\text{CDCl}_3$ ; TMS reference (7).

#### 2. Proton NMR spectrum

The proton NMR spectrum of ivermectin  $\text{CDCl}_3$  was recorded on a Bruker AM-300 (300 Mhz) spectrometer using TMS reference (7). Figure 2 shows the spectrum and Table II lists some proton chemical shifts.

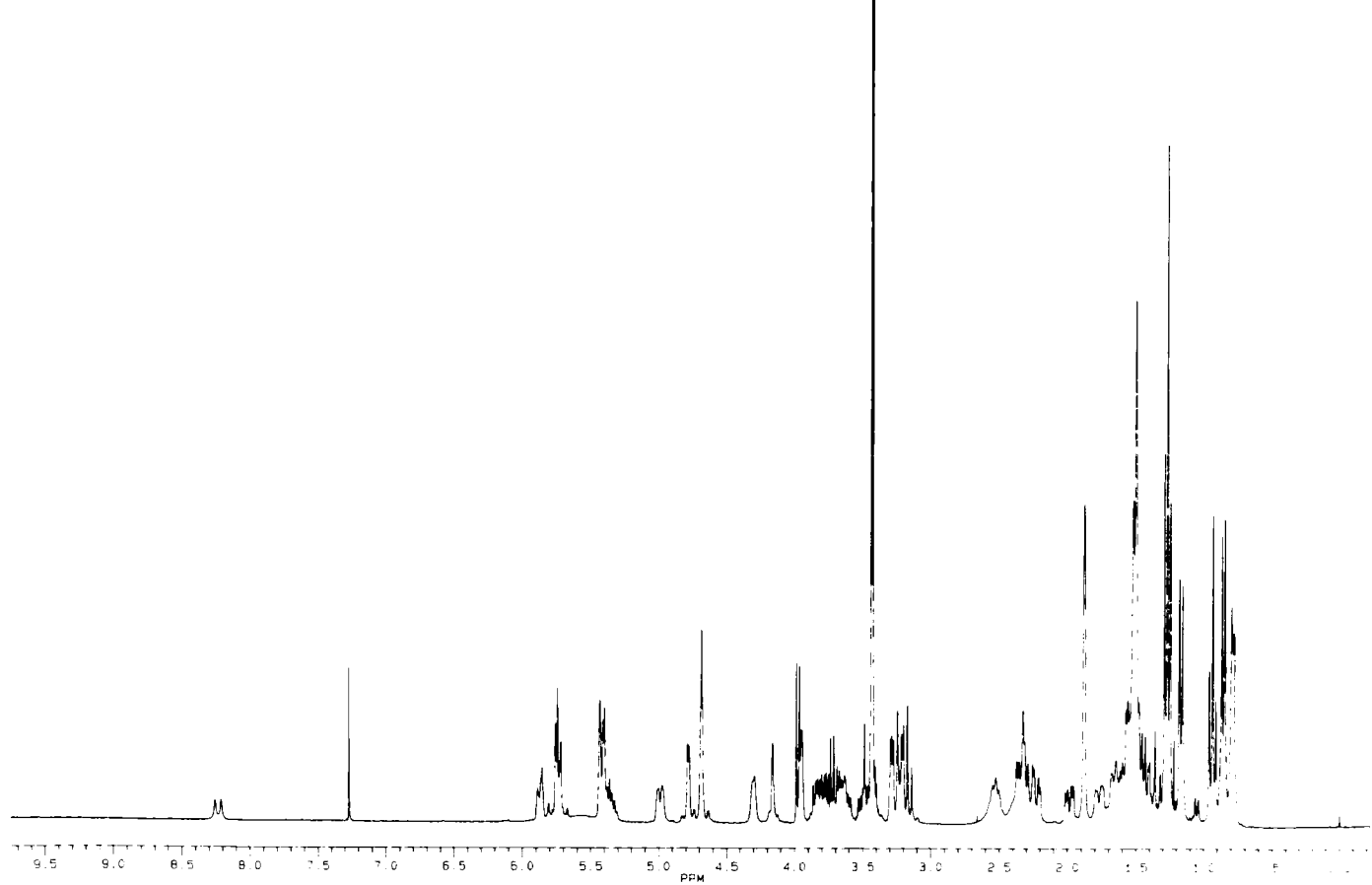




TABLE I. Carbon-13 chemical shifts of ivermectin (7)









Shift $\delta^{13}\text{C}$ (ppm)	Assignment; Carbon Position <sup>a</sup>	Shift $\delta^{13}\text{C}$ (ppm)	Assignment; Carbon Position <sup>a</sup>
173.7	C(1)	67.2	C(5'') & C(17 or 19)
139.6	C(8)	56.5	} -OCH <sub>3</sub> 's at C(3' & 3'')
138.0	C(11)	56.4	
137.8	C(4)	45.8	C(2)
135.0	C(14)	41.2	C(20)
124.7	C(10)	39.8	C(12)
120.4	C(9)	36.9	C(16)
118.4	C(3)	35.8	C(22)
118.1	C(15)	35.5	C(26)
98.5	C(1'')	34.5	} C(18), C(2'), C(2'')
97.5	C(21)	34.3	
94.8	C(1')	34.1	
81.8	C(13)	31.2	C(24)
80.5	C(4)	28.1	C(23)
80.4	C(7)	27.3	C(27)
79.4	} C(6), C(3')	20.2	C(12a)
79.3		19.9	C(4a)
78.2	C(3'')	18.4	C(6'')
76.5	C(25)	17.7	C(6')
76.1	C(4'')	17.4	C(24a)
68.7	C(5'')	15.1	C(14a)
68.4	C(17 or 19)	12.4	C(26a)
68.2	C(8a)	12.1	C(28)
67.7	C(5)		

<sup>a</sup>See Structure VIII for numbering

## B. Infrared Spectrum

Figure 3 shows the infrared absorption spectrum of ivermectin recorded as a KBr pellet on a Nicolet 7199 Fourier Transform spectrometer at a resolution of 2 cm<sup>-1</sup>. Some band assignments of this spectrum are listed in Table III (8).

TABLE II. Proton NMR chemical shifts of ivermectin (7)

ppm	Pattern	Assignment <sup>a</sup>
0.80	d(broad)	24-CH <sub>3</sub>
0.87	d	26-CH <sub>3</sub>
0.93	t	28(CH <sub>3</sub> )
1.18	d	12-CH <sub>3</sub>
1.27	d }	5'-CH <sub>3</sub> & 5''-CH <sub>3</sub>
1.30	d }	
1.51	s	14-CH <sub>3</sub>
1.78	d(broad)	18- <u>eq</u>
1.88	s	4-CH <sub>3</sub>
1.99	dd	20- <u>eq</u>
2.52	m	12(  CH)
3.17	t	4''(  CH)
3.42	s }	OCH <sub>3</sub> 's
3.44	s }	
3.95	s(broad)	13(  CH)
3.98	d	6 (  CH)
4.17	s	7-OH
4.30	d(broad)	5(  CH)
4.69	m	8a-(CH <sub>2</sub> )
4.78	d	1'(  CH)
4.98	d(broad)	15(=CH-)
5.34	m	19 (  CH)
5.40	d	1'' (  CH)
5.43	s(broad)	3 (=CH-)
5.74	m	10,11 (-HC=CH-)
5.87	d(broad)	9 (=CH-)

N.B. The broadened doublet at  $\delta$  8.23 ppm is HCONH<sub>2</sub> solvent in the drug lot

<sup>a</sup>See structure VIII for numbering

### C. Mass Spectrum

The electron-impact ionization mass spectrum of ivermectin is shown in Figure 4. This sample of the drug, which contained 81.5% of the a-component and 7.86% of its b-homolog, was examined using a Finnigan MAT 212 instrument (9). The direct probe operating conditions included a 90-eV ionizing potential and 3-kV accelerating potential.

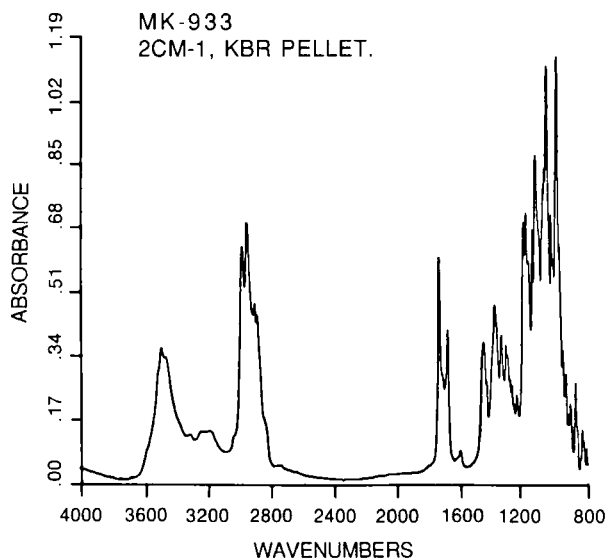


Fig. 3. Infrared Absorption Spectrum of Ivermectin (8).

TABLE III. Band assignments of infrared spectrum of ivermectin (8)

Wavenumber, $\text{cm}^{-1}$	Assignment
3485	OH stretch
3052	CH stretch olefin
2963	asym. $\text{CH}_3$ stretch
2919	asym. $\text{CH}_2$ stretch
2860	sym $\text{CH}_3$ stretch
2852	sym $\text{CH}_2$ stretch
1715	C=O
1680	C=C stretch
1470	$\text{CH}_2$ bend
1390	{ $\text{CH}_3$ sym bend
1350	
1320	
1200	
1200	lactone C-O stretch
1100-950	{ C-O stretch of
	alcohol
	acetal
	ether

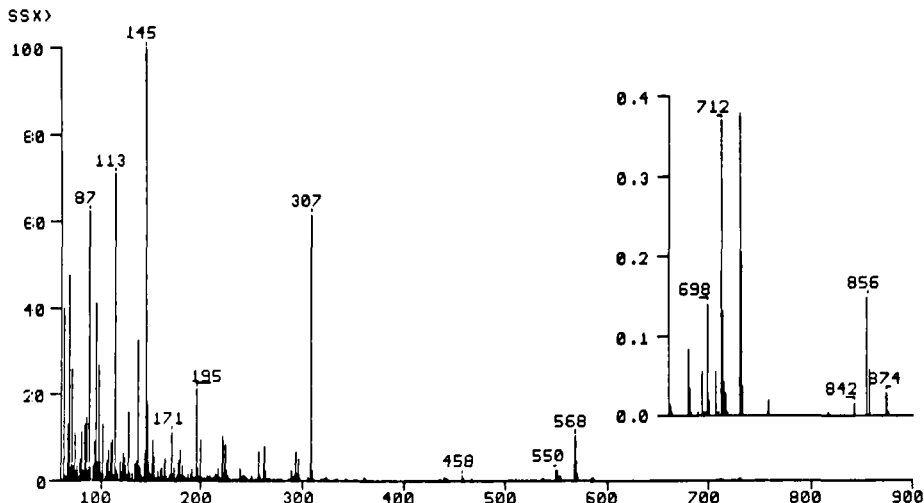


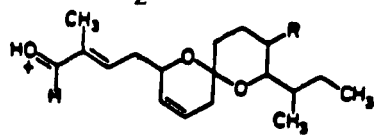
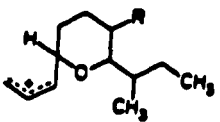
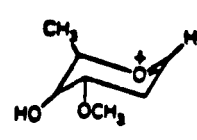
Fig. 4. Electron-Impact Mass Spectrum of Ivermectin (9).

Table IV presents a listing of pertinent ions arising from the a-component and their structural assignments (9). A low-intensity molecular ion is observed at  $m/z$  874, and an ion corresponding to the loss of water is seen at  $m/z$  856. Fragment ions can also be seen resulting from the loss of one and of both of the sugar rings, and the corresponding loss of water from these ions. Intense signals arising from the sugar rings and from the loss of methanol from them are observed at  $m/z$  145 and 113, respectively. Tandem chemical ionization mass spectrometry/mass spectrometry combined with preparative scale HPLC isolation has been used for the confirmatory qualitative determination of ivermectin in cattle tissue (10).

#### D. Ultraviolet Absorption Spectrum

Figure 5 presents the ultraviolet absorption spectrum of the pure a-homolog of ivermectin in methanolic solution. This drug exhibits one absorption band at  $\lambda_{\max}$  244 nm [ $\epsilon = 3.01 \times 10^4$  L/(mole-cm) (11)] with shoulders on both the high- and low-energy sides at 238 nm [ $\epsilon = 2.71 \times 10^4$  L/(mole-cm) (11)] and 254 nm. This methanolic solution is transparent at  $\lambda > 270$  nm. No significant solvent effects have been observed on this absorption spectrum. Because the sites of unsaturation and the oxygen-bearing functional groups are common to both the a- and b-components of the drug, this absorption spectrum is independent of homolog ratio.

TABLE IV. Mass spectrometric ions arising from ivermectin  $H_2B_{1a}$  (9)

m/z	Structure
874	M
856	M-18 ( $H_2O$ )
730	M-144 ( $C_7H_{12}O_3$ ); corresponds to monosaccharide of $H_2B_{1a}$
712	730-18 ( $H_2O$ )
586	M-288 ( $C_{14}H_{24}O_6$ ); corresponds to aglycone of $H_2B_{1a}$
568	586-18 ( $H_2O$ )
550	568-18 ( $H_2O$ )
307 (293) <sup>a</sup>	 <p>307</p> <p>R = <math>CH_3</math></p>
195	 <p>195</p> <p>R = <math>CH_3</math></p>
145	 <p>145</p>
113	145-32 ( $CH_3OH$ )

<sup>a</sup>Analogous ion (14 units lower in mass) arising from  $H_2B_{1b}$

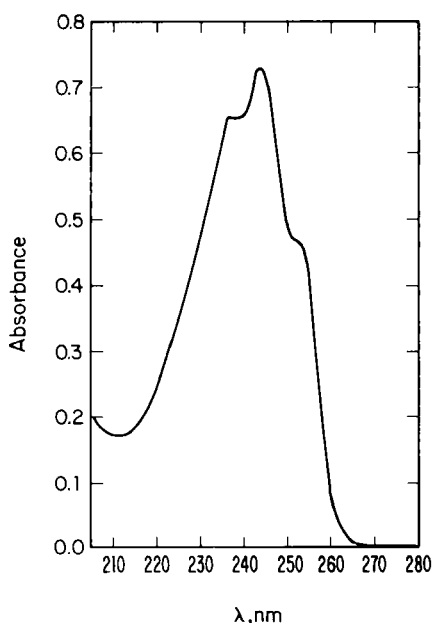


Fig. 5. Ultraviolet Absorption Spectrum of the a-Component of Ivermectin. Solvent: Methyl Alcohol. Concentration:  $2.1 \times 10^{-5}$  M.

### III. SOLUBILITY

Although ivermectin contains two sugar rings and the two polar hydroxyl groups of the dihydroxycyclohexene ring, this drug is nevertheless insoluble in water. Its aqueous solubility at room temperature is only on the order of  $<1 \mu\text{g/mL}$  (12). Although it is not soluble in water, it does dissolve ( $>20\%$  w/v) in other protic solvents such as 1-butanol, methanol, and 1-hexanol; and, its aqueous insolubility is not contrasted by a general lipophilic solubility: although insoluble in water, it is also insoluble ( $<0.1\%$  w/v) in the nonpolar aprotic solvents cyclohexane, n-hexane, and isooctane. The solubility of ivermectin at room temperature in a selection of 23 organic solvents, with representative polar and nonpolar members including several member of hydroxylic, of halogenated, and of aprotic and basic solvent classes, are presented for comparison by functional group in Table V (13).

TABLE V. Solubility of ivermectin at room temperature, mg/mL

<u>Hydrocarbons</u>		<u>Alcohols</u>		<u>Halogenated Solvents</u>	
toluene	63	1-butanol	330	1,2-dichloroethane	440
xylenes	31	methanol	220	chloroform	310
cyclohexane	0.77	1-hexanol	220	dichloromethane	290
n-hexane	0.69	ethanol (95%)	97		
isooctane	0.45	2-propanol	70		
<u>Ethers</u>		<u>Ketones</u>		<u>Miscellaneous</u>	
p-dioxane	430	methylethyl- ketone	320	N,N-dimethyl- formamide	510
tetrahydro- furan	390	acetone	81	ethyl acetate	240
diethyl ether	61	acetyl- acetone	278	dimethyl sulfoxide	220
				acetonitrile	40

As a group, the solubility of ivermectin in hydrocarbons is markedly less than in any of the other groups examined, and within this group the effect of unsaturation is significant: The aromatic solvents dissolve much more drug than the saturated (acyclic or alicyclic) members. Among the ethers, the solubility in the cyclic ethers is greater than in linear solvents. In any series, no significant correlation with solvent polarity is apparent except among the hydrocarbons, in which a rank order of solubility with dipole moment is evident.

Three representative solvents which have been used in analytical procedures for the extraction of ivermectin from different samples include methanol (220 mg/mL) for application to medicated feeds (14), ethyl acetate (240 mg/mL) for plasma samples (15), and methylene chloride (290 mg/mL) in the metabolism studies (16).

Figure 6 presents the solubility of ivermectin in mixed ethanol-water solvents over the range 10% to 100% ethanol at 18°C; these solvent mixtures have been considered as pharmaceutical formulation vehicles. At ethanol concentrations <20%, ivermectin is practically insoluble ( $\leq 0.02$  mg/mL) and over the range 20%-60% ethanol, ivermectin solubility increases in nearly a logarithmic fashion. As shown in Figure 6, ivermectin exhibits a maximum solubility in this solvent system of ca. 100 mg/mL in solvent mixtures composed of 80%-90% ethanol and decreases to 70 mg/mL in anhydrous ethanol (17).

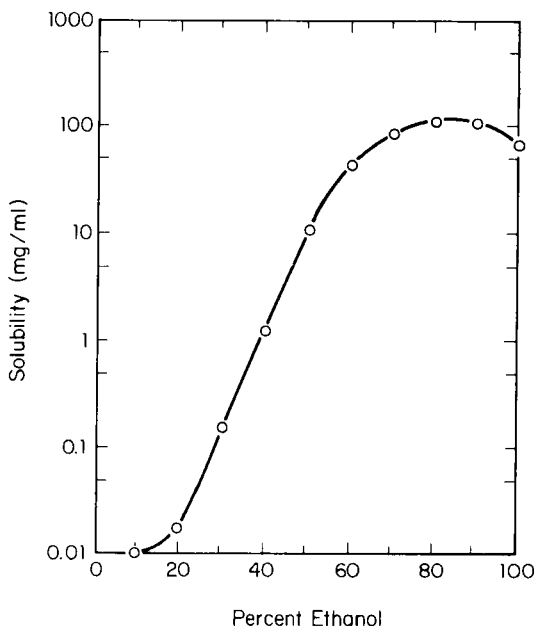


Fig. 6. Solubility of Ivermectin in Ethanol-Water Mixtures at 18°C.

#### IV. DISTRIBUTION RATIOS

Because of the unique solubility properties of ivermectin (Table V), liquid-liquid partitioning affords a wide range of combinations of various solvent pairs for use in sample preparation in analytical methods. Liquid-liquid distribution ratios were determined by equilibrating a known amount (2 mg) of ivermectin in various solvent systems consisting of 10.00 mL of the organic solvent and 10.00 mL of water. The two phases were separated after partitioning and analyzed for ivermectin using reversed-phase high-pressure liquid chromatography (18).

The distribution ratios measured,  $D$  (org/aq), were  $> 100$  for each of 12 organic solvents examined. Hence, ivermectin partitions quantitatively into organic solvents from aqueous unbuffered solution. (Because the neutral drug ivermectin contains neither a strongly acidic nor strongly basic functional group, pH of the aqueous phase would not be expected to have a significant effect on these distribution ratios.) Some representative results are: ethyl acetate ( $\log D = 4$ )  $>$  chloroform ( $\log D = 3$ )



> cyclohexane  $\approx$  isooctane ( $\log D = 2$ ). Note that although ivermectin is insoluble ( $<0.1\%$ ) in the saturated hydrocarbons cyclohexane and isooctane, it nevertheless partitions into these solvents from aqueous solution because of its extremely low solubility in the latter solvent. Two examples of the use of these partitioning properties of ivermectin in analytical applications are the extraction techniques used in the analytical methods reported for the determination of ivermectin in tissue samples (19,20). In these procedures the analyte is extracted into isooctane (19) or methylene chloride (20) by partitioning the samples between the organic extraction solvent and an aqueous-acetone mixed solvent.

The above is one example of the use of ivermectin partitioning properties for analyte isolation during sample preparation in analytical methods. These properties of ivermectin allow great flexibility in developing analytical procedures because ivermectin partitioning behavior can be inverted by the use of a mixed polar phase containing a polar organic solvent modifier as cosolvent with water. Using three-component solvent systems, ivermectin can be quantitatively partitioned into the organic or the aqueous phase by selection of the appropriate component ratios. For example, with acetonitrile/water/hexane the drug is distributed into the polar phase when the relative amounts of these solvents are 4:1:5 (v:v:v) and into the hydrocarbon phase when the relative amount of water is increased (and the polar organic decreased) to the ratio 1:4:5 (v:v:v).

Examples of these distribution ratio measurements are presented in Figure 7, to illustrate the use of (i) acetone and (ii) acetonitrile as modifiers to regulate solvent-solvent distribution between (a) water and isooctane and (b) water and hexane, respectively. One example of the use of these distribution ratio inversions appears in the analytical method for the determination of ivermectin in tissues (19): after extraction into isooctane by partitioning against water containing 50% (v/v) acetone [ $\log D (\text{org/aq})=1$ : Figure 7], the drug is then back-extracted in this analytical method into neat acetonitrile partitioned against hexane ( $\log D (\text{org/aq})=-2$ ; Figure 7) and finally back into hexane by using a water-acetonitrile (4:1) mixed solvent for this partitioning step [ $\log D (\text{org/aq})=2$ : Figure 7].

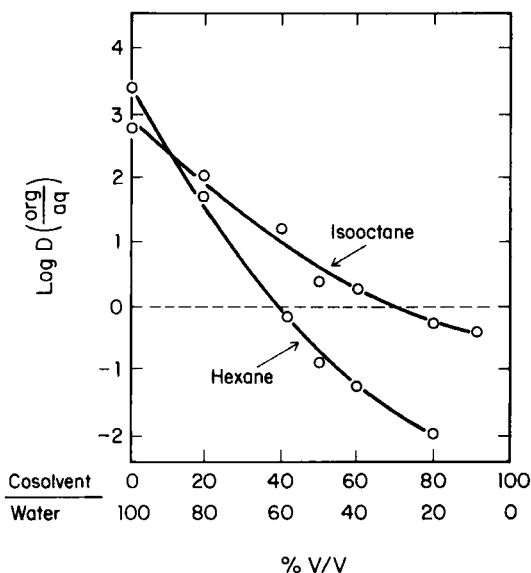


Fig. 7. Distribution Ratios of Ivermectin Between Organic Solvents and Mixed Aqueous-Polar Organic Solvent Phase: Distribution as a Function of Aqueous Phase Composition.

#### Figure Legend:

<u>Line</u>	<u>Cosolvent of Aqueous Phase</u>
Isooctane	Acetone
Hexane	Acetonitrile

## V. CHROMATOGRAPHIC PROPERTIES

### A. TLC

Ivermectin can be observed with intermediate  $R_f$ 's on silica gel plates in a large number of solvent systems. Representative of these are: (a)  $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{OH}$ -con.  $\text{NH}_4\text{OH}$  (90:9:1.5),  $R_f = 0.65$  and (b)  $\text{Et}_2\text{O}$ - $\text{CH}_3\text{OH}$ - $\text{HOAc}$  (90:9:1.5),  $R_f = 0.74$ . Each of these systems provides a single spot containing both ivermectin homologs. In addition, the former solvent resolves this drug from its 2-epimeric product of alkaline isomerization (see STABILITY section below). On  $\text{C}_{18}$

reversed-phase TLC plates, the major (a) component of ivermectin can be seen at  $R_f$  0.42 using a  $\text{CH}_3\text{OH}-\text{H}_2\text{O}$  (90:10) solvent mixture and at  $R_f$  0.44 using  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$  (90:10). This latter solvent also clearly resolves the drug from its monosaccharide ( $R_f$  = 0.53) and aglycone ( $R_f$  = 0.66) degradation products of acidic hydrolysis (STABILITY section).

## B. Column Chromatography

Ivermectin can be separated from many interferences which are adsorbed on a conventional gravity-fed alumina (activity III) column when charged to this column in methanolic solution. This chromatographic behavior has been used to isolate ivermectin efficiently from many extraneous components of feed samples. The analyte is not adsorbed on this stationary phase and does not undergo any chemical reaction during this analytical chromatographic pretreatment (14). Ivermectin can also be isolated by column chromatography on Florisil (an activated magnesium silicate). The drug is adsorbed on this substrate from chloroform solution and, after further elution of extraneous compounds with additional chloroform, the analyte is recovered from the Florisil in a mixed chloroform-ethyl acetate (3:1) eluent. This chromatographic isolation step is effective for the preparation of plasma samples for analysis (15). A tissue residue analytical method based on HPLC-RIDA also affords an example of the use of Silica Sep-Pak cartridges in analyte isolation (20).

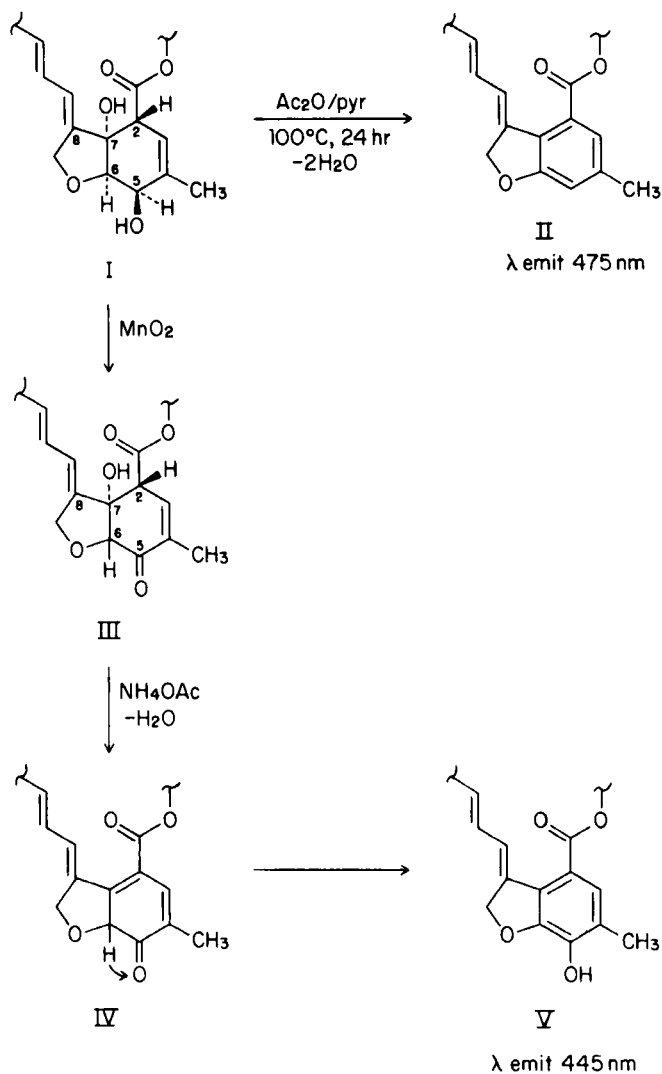
## C. HPLC

Both reversed-phase and normal-phase chromatographic conditions have been established for the measurement of ivermectin. Typical of the reversed-phase systems are a ZORBAX ODS (DuPont) column stationary phase with a mixed solvent mobile phase of acetonitrile-methyl alcohol-water (53:35:7) (14,21). A representative chromatogram obtained using these conditions is presented in Figure 8; the details of the instrumental conditions used are listed in that figure caption. This chromatogram illustrates the efficient resolution of the two homologous members of ivermectin furnished by this chromatographic system. This figure reveals that more polar homolog (b) is less strongly retained than the a compound on this nonpolar stationary phase (22). For quantitative analysis, the  $\Delta^2$  isomer of the a-homolog (VII; Scheme II) can serve as a convenient internal standard (21). Prichard,

et al. (23) have also used a diacetylated derivative of the a-component of ivermectin at the 4"- and 5-hydroxy functional groups as an internal standard for use on a 5- $\mu$  C<sub>18</sub> reversed-phase Radialpak A column (Waters Associates). The monosaccharide of the a component has also been used as an internal standard with fluorescence derivatization (20). One alternative set of chromatographic conditions would use acetonitrile-methyl alcohol-water in the volume ratio 39:26:35 on the same ZORBAX ODS column (16), and an HPLC-RIDA method used for the determination of ivermectin in tissues uses a mobile phase of acetonitrile-methanol-water (49.2:32.8:1.8 v/v/v) on the ZORBAX ODS column (20). A third set of reversed-phase conditions uses an acetonitrile-methyl alcohol-water (52:26:22) mixed mobile phase on a (Dupont) ZORBAX C-8 stationary phase (24). A normal-phase system has been reported using n-hexane-tetrahydrofuran-methyl alcohol-dimethylsulfoxide (75:25:4:2) on a uBondapak NH<sub>2</sub> (Waters) stationary phase (25). These conditions do not resolve the two compounds.

## VI. DERIVATIZATION

The potency of ivermectin, which permits the use of low dosage levels (e.g., 0.2 mg/kg) also requires sensitive analytical procedures. Accordingly, several derivatives have been developed to increase sensitivity for HPLC detection. For instance, an intensely fluorescent derivative has been produced by aromatization of the C(2)-C(7) ring. Ivermectin per se is not intrinsically fluorescent. The two hydroxyls [on C(5) and C(7)] of the dihydroxycyclohexene ring of ivermectin (I) are each adjacent to a trans proton [on C(6) and C(2), respectively]. Heating with acetic anhydride in pyridine causes the elimination of these two moles of water, furnishing an aromatic C(2)-C(7) ring, which, in conjugation with the C(8)-C(11) diene, is responsible for the fluorophore (II). This dehydration reaction to the fluorescent aromatic derivative is shown in the partial structures of Scheme I. The mechanism of this analytical reaction involves acetylation of the hydroxyl groups prior to dehydration. Compound II emits an intense fluorescence at 475 nm under excitation at 360 nm. It is the basis of a sensitive HPLC analytical method which uses fluorescence detection after preliminary sample preparation by liquid-liquid partitioning and conventional gravity-fed column chromatography. This fluorophore has been applied to plasma samples obtained from animals dosed with the



Scheme I  
Fluorogenic Derivatization Reactions of Ivermectin (I) via  
Aromatization  
[Partial Structures]

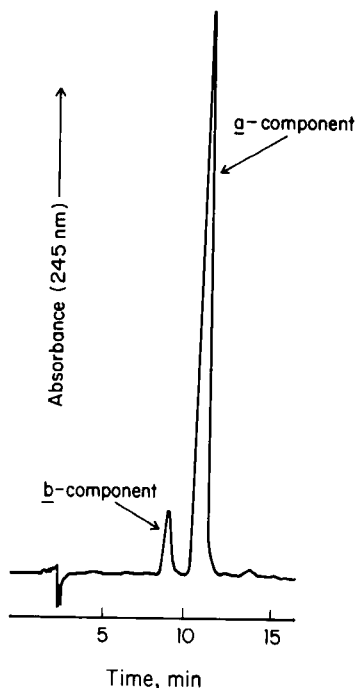


Fig. 8. Representative Reversed-Phase HPL Chromatogram of Ivermectin Illustrating the Resolution of Its Two Components. Drug Composition is 91% a-Component and 9% b-Component; Concentration: 14.1  $\mu\text{g/mL}$  in Methyl Alcohol; 100  $\mu\text{L}$  Injected.

Chromatographic Conditions: Column: Zorbax ODS (DuPont), 4.6 x 250 mm; Mobile Phase:  $\text{CH}_3\text{CN}:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (53:35:7); Flow Rate: 1.2 mL/min; Ambient Temperature; Detection: UV Absorbance at 245 nm; 0.08 aufs.

drug and which contain as little as 0.2 ng ivermectin per milliliter of plasma in a 5-mL sample (15,26). This fluorescent derivative (II) has also been applied in tissue analysis (liver, fat, muscle, and kidney) via a more rapid derivatization reaction which uses an N-alkylimidazole nucleophilic acetylation catalyst in lieu of pyridine (19).

Ivermectin has also been derivatized to a different aromatic derivative which retains the -OH functional group on C(5) (27). This derivative (V) exhibits an intense emission centered at 445 nm under excitation at 361 nm. The analytical reactions used to generate V are also shown in Scheme I: in this method the drug is oxidized with

MnO<sub>2</sub> to its 5-ketone intermediate (III) which is converted to the fluorescent derivative (V) by treatment with NH<sub>4</sub>OAc in ethyl alcohol. As shown in Scheme I, the derivative (V) is the enol form of keto IV derived from III by the loss of water at C2(H) and C7(OH). This derivative (V) is also electroactive, exhibiting characteristic phenolic electroactivity (28) which can be used in analytical applications with sensitive electrochemical detection of HPLC. Underivatized parent ivermectin is electroinactive.

## VII. STABILITY

### A. Intrinsic (Solid State) Stability

Drug stability studies have demonstrated that neat ivermectin--in the absence of extraneous reactants and impurities--is a stable molecule in its crystalline powdered state. No drug degradation has been found following examination of several drug batches after exposure to 37°C for 1-1/2 years, 40°C for 1/2 year and 50°C for three months. These analyses were performed by a stability-indicating HPLC analytical technique (vide infra).

### B. Stability in Solution

#### 1. Aqueous Solvents

Because ivermectin contains many functional groups, it can participate in a wide variety of reactions in solution. It is unstable both in acidic and in basic solution, undergoing different reactions in these two media. Figure 9, which shows the rate of degradation as a function of pH, reveals the pronounced acceleration of degradation in solution with both increased acidity and basicity. The optimum pH for solution stability as indicated in this figure is pH 6.3. The ordinate of Figure 9 presents the rate of reaction in units of first-order rate constants. For these data, both total acidic and basic hydrolysis rates were fit to first-order kinetic decay as a function of pH (29). The initial ivermectin concentration was  $5 \times 10^{-3}$  mg/mL, the reaction solvent was 15% (v/v) ethyl alcohol in water, the reaction temperature was 50°C, and the apparent pH was established with phosphate buffers (0.1 M). This figure shows the results obtained over a narrow range of less than two pH units; hydrolysis proceeds much more rapidly at both ends beyond this range, and the minimum at pH 6.3 results from the tradeoff among the several modes of both

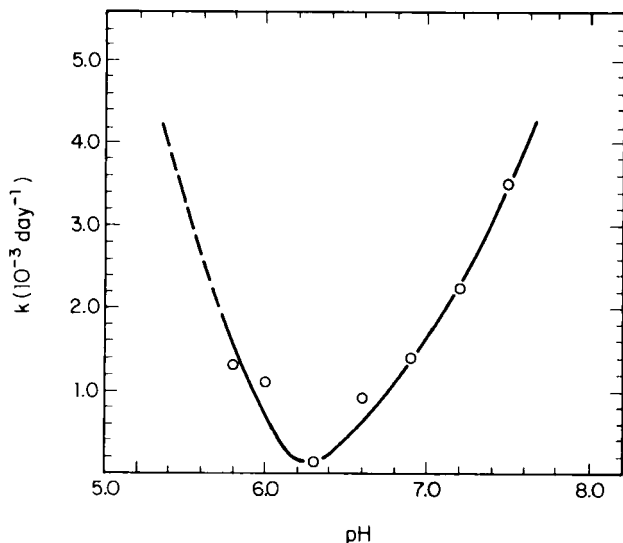


Fig. 9. pH-Reaction Rate Profile for the Hydrolysis of Ivermectin in Solution. Reaction Solvent: 15% (v/v) Ethyl Alcohol in Water. Reaction Temperature: 50°C; initial ivermectin concentration:  $5 \times 10^{-3}$  mg/mL

a. Acidic Hydrolysis. Typical of similar compounds, the acidic degradation of ivermectin results in hydrolysis of the two sugar rings to yield the monosaccharide and aglycone degradates as the predominant products. These compounds were identified by comparison of their retention times to those of the corresponding authentic compounds. Figure 10 shows the reaction profile for the acid hydrolysis of ivermectin in 0.5 M HCl solution (29). This figure shows the degradation of the a-component of ivermectin (which obeys first-order kinetic decay under these conditions) and the concomitant relative rates of appearance of the monosaccharide and aglycone major degradates. As indicated in this figure, the monosaccharide is formed much more rapidly than the aglycone, but this latter compound eventually becomes the predominant product at times later than shown in the figure. As the reaction proceeds, the monosaccharide serves as an intermediate and undergoes cleavage of the second sugar ring to yield the aglycone. Accordingly, the concentration of the monosaccharide reaches a maximum and then decreases in this acidic medium.



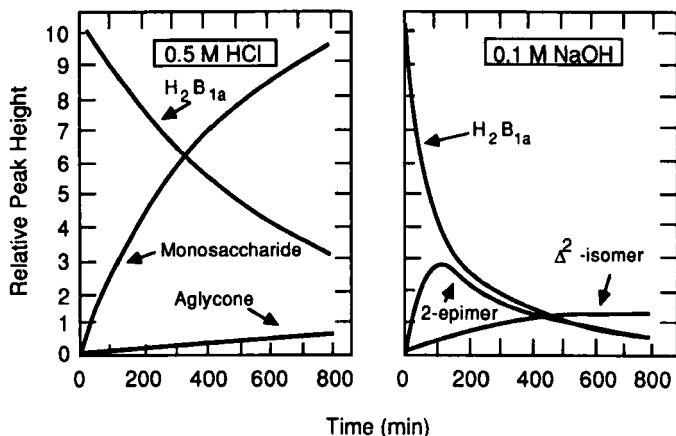
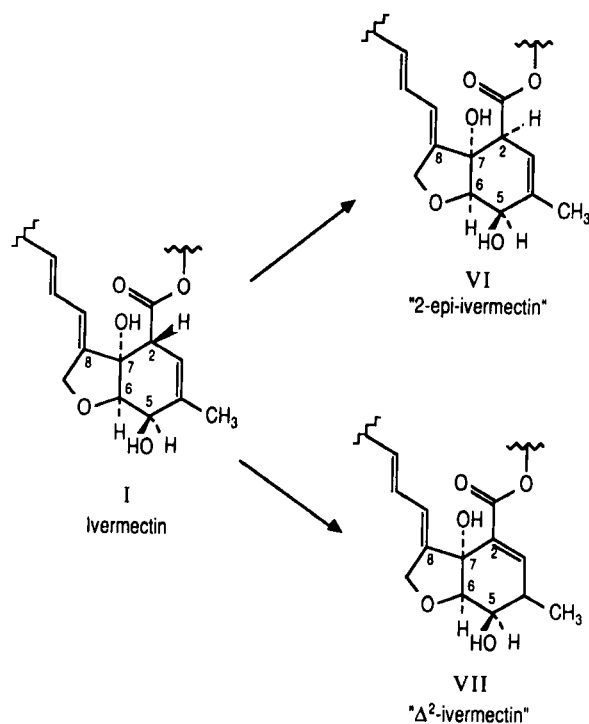


Fig. 10. Reaction Profiles for the Acidic (0.5 M HCl) and Basic (0.1 M NaOH) Hydrolyses of Ivermectin (a-Component) (29).

b. Basic Hydrolysis. Ivermectin can undergo isomerization in basic solution because of the weak acidity of the allylic proton on C(2). The two products of basic isomerization, shown in Scheme II, can be derived from the delocalized carbanion resulting from dissociation of the proton at C(2). Reprotonation of the anion from the opposite face of the molecule at C(2) generates the epimeric product "2-epi-ivermectin" (VI). Alternatively, reprotonation at C(4) generates the structural isomer " $\Delta^2$ -ivermectin" (VII). The 2-epimer (VI) of ivermectin was identified by comparison of its retention time to that of the authentic compound, and the structure of the  $\Delta^2$  isomer (VII) was determined by 300 MHz nmr spectroscopy. Figure 10 shows the time course of the degradation profile of ivermectin in 0.1 M NaOH (21,29).

The high-pressure liquid chromatographic system described in Figure 8 can be used as the basis of a stability-indicating analytical measurement for parent ivermectin in the presence of its four predominant products of acid and base hydrolysis. That this system resolves the drug from these compounds is illustrated in Table VI, which lists the relative retention times (RRT's) of the four main degradates relative to the retention time of the a-homolog of ivermectin (30).



**Scheme II**  
**Alkaline Hydrolysis Products**  
 of Ivermectin (I): "2-epi-ivermectin"  
 (VI) and " $\Delta^2$ -ivermectin" (VII) [Partial Structures]

**TABLE VI. Relative HPLC retention times (RRT's) of predominant ivermectin acidic and basic degradates<sup>a</sup>**

Compound <sup>b</sup>	Relative Retention Time (RRT)
aglycone	0.45
monosaccharide	0.7
ivermectin	1.00
2-epimer (VI)	1.12
$\Delta^2$ -isomer (VII)	1.25

<sup>a</sup>Under chromatographic conditions described in Figure 8

<sup>b</sup>These compounds are all the a-homologs of ivermectin

## 2. Nonaqueous solvents

Although ivermectin exhibits hydrolysis under  $\text{OH}^-$ - and  $\text{H}^+$ -attack in aqueous solution, it is stable in the dark in aliphatic hydroxylic polar solvents such as methyl alcohol, propylene glycol, and a mixture of the isomers of glycerol formal, 4-hydroxymethyl-1,3-dioxolane and 5-hydroxy-1,3-dioxane (31). Known solutions of ivermectin in these solvents can be stored as analytical reference standards at ambient conditions for several months with no degradation.

## 3. Other Degradation

Although ivermectin is stable in the dark in aliphatic hydroxylic solvents, it is photolabile in aromatic solvents. The rates of photodegradation (fluorescent and ultraviolet irradiation;  $25^\circ\text{C}$ - $30^\circ\text{C}$ ) of ivermectin as a function of solvent increase in the order  $\text{CH}_3\text{CN} \approx \underline{\text{n}}\text{-C}_8\text{H}_{18} \approx \text{EtOH} < \phi\text{-CH}_3 \approx \phi\text{CH}_2\text{OH} < \text{CH}_3\text{COOC}_2\text{H}_5$  (32).

The aliphatic solvents acetonitrile, n-octane, and ethyl alcohol are the most transparent of this group and exhibit the slowest reaction rates independent of solvent polarity and hydroxylic character. The aromatic solvents ( $\pi^* \leftarrow \pi$  absorption bands) accelerate the reaction relative to the aliphatic solvents, with rates which are also independent of solvent polarity and the hydroxyl group. These data demonstrate that aromatic ( $\pi^* \leftarrow \pi$ ) and carbonyl ( $\pi^* \leftarrow \text{n}$ ) absorption transitions sensitize the photodecomposition of the drug and suggest an energy-transfer mechanism. The photoproducts are typically geometric isomers at the C(8)-C(9) and C(10)-C(11) olefins.

Ivermectin is subject to oxidative degradation at the 8a-methylene site to produce mixtures of 8a-hydroxy, its corresponding open form the 8a-aldehyde, and other oxidation products. This oxidation, which occurs in nonpolar solvents and in aqueous surfactant solutions, is enhanced by transition metal ions and free-radical initiators. It is the predominant mode of degradation under typical neutral conditions. The 5-OH functional group can also produce a 5-keto oxidative degradate of ivermectin (III; Scheme I).

## VIII. SYNTHESIS

The preparation of the semi-synthetic drug ivermectin was reported by Chabala et al. (5). The general structure of the precursor natural product avermectin mixture of

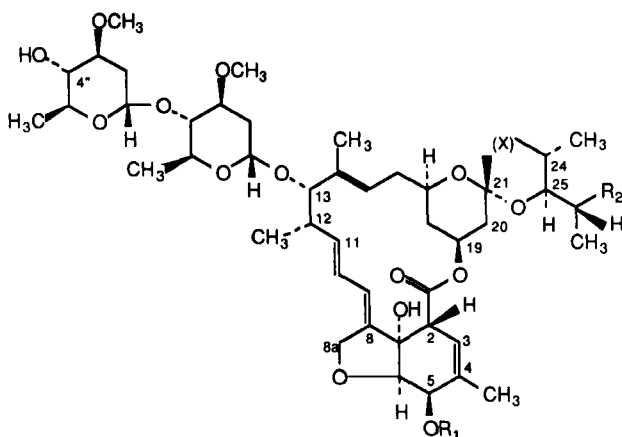
compounds is shown as structure VIII. These workers determined that, because of bioavailability and efficacy correlations, the optimal ivermectin drug structure was that possessing the conformation of the saturated carbons C(22) and C(23), and lacking the -OH on C(23). Accordingly, the starting materials selected were the avermectin-1 series of compounds possessing the C(22)-C(23) double bond for treatment. The equal biological activity of the a- and b-homologs obviated their difficult separation.

This selective hydrogenation of only one of five olefins in the precursor was achieved using Wilkinson's homogeneous hydrogenation catalyst,  $(\text{Ph}_3\text{P})_3\text{RhCl}$ . This selectivity results from the fact that the C(22)-C(23) double bond is the only one of the five which is only cis-substituted. The starting material (VIII) was treated with  $(\text{Ph}_3\text{P})_3\text{RhCl}$  in benzene at 25°C under  $\text{H}_2$ , resulting in the stoichiometric consumption of one mole of  $\text{H}_2$  after 20 hours. After solvent washing, the product was purified on silica gel to give ivermectin in 85% yield. The selectivity of this synthesis was demonstrated by the presence in the product of only 2% of the starting material and 3% of the overhydrogenated tetrahydro product mixture 3,4,22,-23-tetrahydroavermectin  $\text{B}_{1a}$  and 3,4,22,23-tetrahydroavermectin  $\text{B}_{1b}$ . Further purification on Sephadex LH-20 furnished a 99%-pure sample of the drug (I). Mrozik, Eskola, and Fisher (33) also accomplished the selective dehydration of the axial C(23)-OH of avermectin  $\text{B}_{2a}$  to afford the C(22)-C(23) double bond of avermectin  $\text{B}_{1a}$  as precursor to the a-homolog of ivermectin (" $\text{H}_2\text{B}_{1a}$ ").

Synthetic techniques have been brought to bear on the preparation of some of the individual structural units that comprise ivermectin. For example, the hexahydrobenzofuran fragment has been synthesized using nitrile oxide chemistry (34) and in a 6-membered ring forming reaction of high stereoselectivity (35). The challenging spiro ketal moiety has been prepared in optically pure form from optically active pure carbohydrate precursors (36); and in 1985 through a chlorohydrin derivative (37).

## IX. PHARMACOKINETICS AND METABOLISM

The intrinsic pharmacokinetic properties of ivermectin are a function of the animal species studied. For example, the biological half-life  $t_{1/2}$  of this drug increases in the order: swine (0.5 day) < dogs (1.8 day) < cattle  $\approx$  sheep (2.8 day) (38). There is also a significant difference in the volume of distribution of ivermectin among species: in cattle, sheep, and dogs, the volume of



## VIII

ComponentA  $R_1 = \text{CH}_3$ B  $R_1 = \text{H}$ a  $R_2 = \text{C}_2\text{H}_5$ b  $R_2 = \text{CH}_3$ Component1 (X) =  $-\text{CH}=\text{CH}-$   
22 232 (X) =  $-\text{CH}_2-\text{C}-$   
22 23  
OH  
H

**Structures of the Naturally-Occurring Avermectin  
Starting Materials**

distribution,  $V_d$ , is 1.9, 4.6, and 2.4 L/kg, respectively. These results were obtained following intravenous administration to these animal species, and the resulting data, shown in Figure 11, were treated by the usual two-compartment open model. Application of a three-compartment model to the data obtained from six cattle also yielded an equal biological half-life  $t_{1/2} = 2.7$  days in this species (39). In this study, the three-compartment (triexponential) model provided a statistically significantly closer fit to the data than the two-compartment (biexponential) treatment in three of the six animals. Another study reported a longer ivermectin half-life of 7.42 days in sheep (23). Snitzerling and Nolan, who studied the binding of ivermectin to plasma protein in cattle, demonstrated that the drug is carried mainly in the plasma (80%) and that this distribution equilibrium between plasma and blood cells remains relatively constant with time (25).

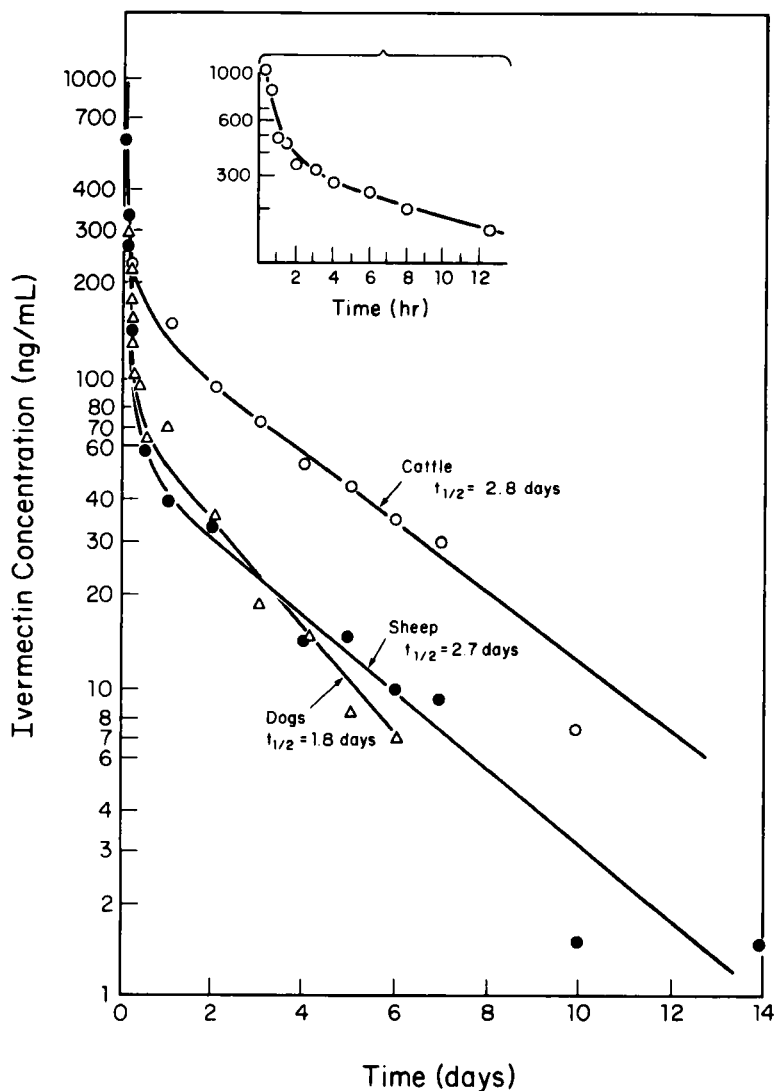


Fig. 11. Pharmacokinetics of Ivermectin. Biexponential Decay of Ivermectin Concentration in Plasma Following Intravenous Administration to Cattle (o), Sheep (●), and Dogs (Δ). Dose Rates - 300 μg/kg Body Weight for Cattle and Sheep; 200 μg/kg for Dogs. The biexponential decay mode for cattle is more clearly evident in the expanded scale insert, which depicts the rapid distribution phase in this species. (Points are averages from: two cattle, four sheep, and five dogs, respectively.)

The metabolic transformations of ivermectin resulting from incubation at 37°C with rat or steer liver microsomes have been studied in vitro by Miwa, et al. (16) using radiolabeled drug, NMR and mass spectrometry as well as reversed-phase radiochromatography and HPLC with direct UV detection on a Zorbax ODS analytical column. The spectral data support the assignment of the two major classes of polar metabolites of ivermectin as the C(24)-hydroxymethyl compounds and their respective monosaccharides. In this study, these workers also described derivatization via trimethylsilylation for mass spectrometric purposes to confirm the presence of the additional hydroxyl group in the metabolites.

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## MINOXIDIL

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- 1.2. Therapeutic Category

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  - 2.1.2. Generic Name
  - 2.1.3. Laboratory Code
  - 2.1.4. Proprietary Names
- 2.2. Formulae
  - 2.2.1. Empirical
  - 2.2.2. Structural
  - 2.2.3. Registry Numbers
- 2.3. Molecular Weight
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  - 7.2.2. Radioimmunoassay (RIA)
- 7.3. General - Thin-Layer Chromatography (TLC)

## 1. Introduction

### 1.1. History

Minoxidil, a piperidinopyrimidine, was initially developed as an antihypertensive agent by The Upjohn Company. The marked hypotensive effect of 2,4-diamino-6-(diallylamino)-S-triazine (1) and the discovery that its corresponding N-oxide metabolite was responsible for the lowering of blood pressure (2,3) eventually led to the development of minoxidil and to its first literature appearance in 1968 (4). Preliminary trials were first described in man in 1969 (5) and after extensive clinical investigations, the drug was marketed in tablet form as Loniten. More recently, the earlier clinical trial evidence of new and increased hair growth (6-8) as well the reports of reversal in male pattern baldness (9,10) created a renewed interest in minoxidil as a therapeutic agent. The compound has, therefore, been reformulated as a topical solution and is presently available in Canada (Rogaine) and in Europe (Regaine) as a hair growth stimulant.

The history, pharmacology and clinical data on minoxidil is well documented in a number of review articles and monographs (11-17).

### 1.2. Therapeutic Category

Minoxidil is a potent antihypertensive drug indicated for the oral treatment of severe symptomatic or organ-damaging hypertension that is not controlled with any other drug or combination of drugs. It acts as a direct-acting peripheral vasodilator that decreases both systolic and diastolic blood pressure by decreasing peripheral vascular resistance. Despite some significant side effects, minoxidil is very effective in reducing hypertension refractory to conventional antihypertensive agents (11,13,17).

Minoxidil is also effective topically as a hair growth stimulant and is indicated for the treatment of alopecia androgenetica (male pattern baldness) (15).

## 2. Description

### 2.1. Nomenclature

#### 2.1.1. Chemical Name

2,4-Diamino-6-piperidinopyrimidine 3-oxide;  
6-(1-piperidinyl)-2,4-pyrimidinediamine 3-oxide;  
6-piperidino-2,4-diaminopyrimidine 3-oxide;  
2,3-dihydro-3-hydroxy-2-imino-6-(1-piperidinyl)-  
4-pyrimidinamine; 6-amino-1,2-dihydro-1-hydroxy-  
2-imino-4-piperidinopyrimidine;

#### 2.1.2. Generic Name

Minoxidil

#### 2.1.3. Laboratory Code

U-10,858, PDP

#### 2.1.4. Proprietary Names

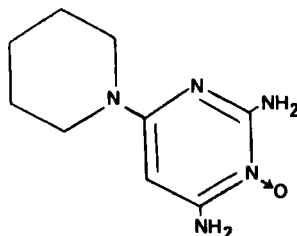
Loniten, Prexidil, Rogaine, Regaine

### 2.2. Formulae

#### 2.2.1. Empirical

$C_9H_{15}N_5O$

#### 2.2.2. Structural



### 2.2.3. Registry Numbers

Chemical abstracts; 38304-91-5

### 2.3. Molecular Weight

209.25

### 2.4. Elemental Composition

C, 51.66%; H, 7.22%; N, 33.47%; O, 7.65%

### 2.5. Appearance, Color and Odor

White or off-white, odorless, crystalline powder.

### 2.6. Patent Information

Neth. Pat. 6,615,385 corresponds to U.S. Pat. 3,382,247 (1967, 1968 to Upjohn) (4); Ger. Offend. 2,114,887 corresponds to U.S. Pat. 3,644,364 (1972 to Upjohn) (18); U.S. Pat. 3,910,928 (1975 to Upjohn) (19). Span. ES. 548,256 (1986) (20); Span. E.S. 548,779 (1986) (21); Span.ES. 549,252 (1986) (22); Span. E.S. 547,627 (1986) (23);

## 3. Pharmaceutical Dosage Forms

Minoxidil is available as a:

- i) compressed tablet; 1, 2 1/2, 5 and 10 mg;
- ii) topical solution; 20 mg/mL in alcohol, propylene glycol and water.

Some dosage forms are not available in certain countries.

## 4. Physico-Chemical Properties

### 4.1. Melting Range

A relatively wide range of melting points has been reported for minoxidil. Reported values include 225°C with decomposition (24), 248°C (decomposition at 259 to 261°C) (25) and 262-264°C (with decomposition) (4). Similar values were obtained with a Mettler FP61 apparatus (248.6°C) and a Gallenkamp melting point apparatus (249-251°C) (values are uncorrected).

### 4.2. Solubility

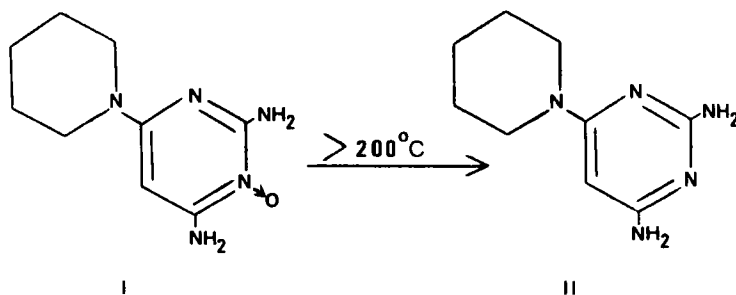
The approximate solubilities of minoxidil in a variety of solvents at ambient temperature is presented in Table 1.

Table 1. Solubility of Minoxidil (ambient temperature)

<u>Solvent</u>	<u>Solubility (mg/mL)</u>
Propylene glycol	75.
Methanol	44.
Ethanol (95%)	29.
2-Propanol	6.7
Dimethylsulfoxide	6.5
Water	2.2
Chloroform	0.5
Acetone	<0.5
Ethylacetate	<0.5
Diethyl ether	<0.5
Benzene	<0.5
Acetonitrile	<0.5

#### 4.3. Stability and Storage

Minoxidil (I) drug substance is a relatively stable substance at room temperature and shows no evidence of significant decomposition. However, exposure to extreme temperature, in excess of 200°C, gives rise to the deoxygenated compound desoxyminoxidil (II) (26).



The degradation of minoxidil in solution (20°C, pH 7.0, phosphate buffer) follows first-order degradation kinetics with a rate constant of  $9.464 \times 10^{-3} \text{ day}^{-1}$  and a calculated activation energy of 11.7 Kcal/mole. Degradation is acid-base dependent showing the greatest stability at pH 5.0 (27).

Minoxidil (tablets and topical solution) should be stored in tightly closed containers and at controlled temperature from 15-30°C (28,29).

#### 4.4. Dissociation Constant

The pKa of minoxidil determined in 0.01M ionic strength buffer is 4.61 (30). This has been confirmed by potentiometric titration (26).

#### 4.5. Partition Coefficient

The octanol-water partition coefficient for minoxidil, based on high performance liquid chromatographic (HPLC) elution or retention times, is 1.24 (31).

#### 4.6. Spectral Properties

##### 4.6.1. Ultraviolet Spectra

The ultraviolet spectra of minoxidil were scanned from 200 to 350 nm with a Shimadzu UV-265 ultraviolet-visible recording spectrophotometer at a concentration of 40  $\mu$ moles/L in methanol (Figure 1), 0.01 N potassium hydroxide (Figure 2) and 0.01 N sulfuric acid (Figure 2) respectively. The spectra in methanol and aqueous potassium hydroxide show almost identical absorption maxima (methanol-230.8, 261.4, and 286.3 nm; 0.01 N KOH-229.7, 261.7 and 288.9 nm). In aqueous acid, a hypsochromic shift to a lower wavelength (281.4 nm) and a corresponding hyperchromic shift was observed.

The absorption maxima, A (1%, 1 cm) values and molar absorptivity values for minoxidil in methanol, 0.01 N KOH and 0.01 N H<sub>2</sub>SO<sub>4</sub> are presented in Table 2. The recorded values are in general agreement with those reported in the literature (24,25).

Table 2. Ultraviolet Spectral Characteristics of Minoxidil

Solvent	$\lambda$ max (nm)	A(1%, 1cm)	$\epsilon$
Methanol	230.8	1687.7	35,315
	261.4	561.1	11,740
	286.3	609.5	12,755
0.01N KOH	229.7	1613.7	33,766
	261.7	516.6	10,792
	288.9	583.6	12,213
0.01N H <sub>2</sub> SO <sub>4</sub>	229.9	1117.6	23,385
	281.4	1114.7	23,164



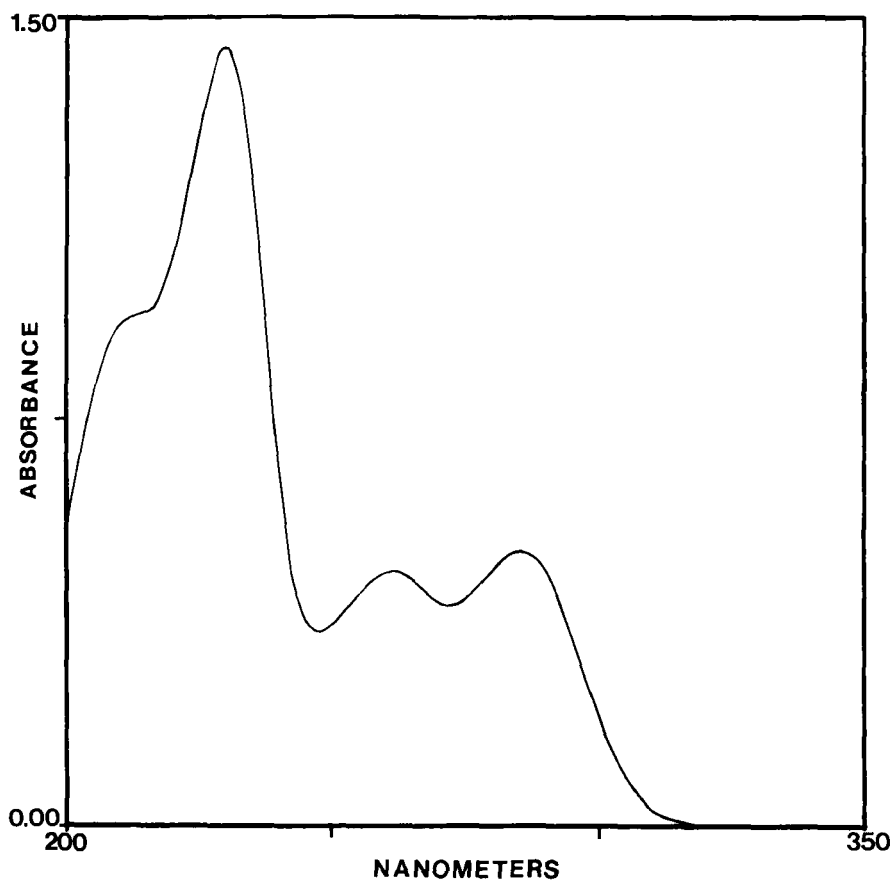


Figure 1. Ultraviolet Spectrum of Minoxidil in Methanol.

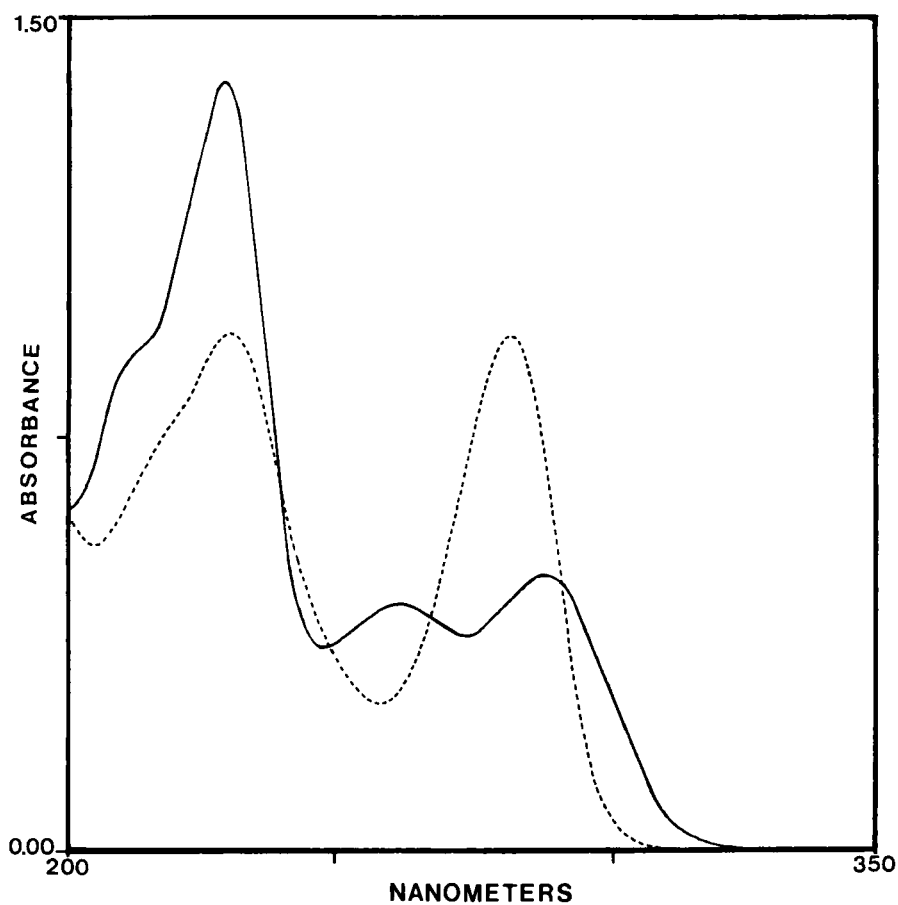


Figure 2. Ultraviolet Spectra of Minoxidil (—, 0.01 N KOH; ----, 0.01 N H<sub>2</sub>SO<sub>4</sub>).

#### 4.6.2. Fluorescence Spectra

The corrected fluorescence spectra for minoxidil in water (25  $\mu$ moles/L) were obtained on a Spex Fluorolog spectrofluorometer scanned from 240 to 550 nm (Figure 3).

Minoxidil exhibits weak fluorescence when excited by ultraviolet light. The excitation and emission maxima occur at about 290 nm and 445 nm respectively.

#### 4.6.3. Infrared Spectrum

The infrared spectrum of minoxidil is shown in Figure 4. The spectrum was obtained with a Beckman AccuLab 4 infrared spectrophotometer from a compressed KBr disc. Structural assignments of some of the characteristic absorption bands are presented in Table 3.

Table 3. Infrared Spectral Assignments for Minoxidil

<u>Frequency (<math>\text{cm}^{-1}</math>)</u>	<u>Intensity</u>	<u>Assignment</u>
3470, 3445, 3430, 3385	M	N-H Stretch
3280, 3040	M(broad)	H-bonded N-H
2975, 2955, 2880	M	aromatic and aliphatic C-H stretch
1650, 1618	S	aromatic C=N stretch
1568, 1485, 1475	S	aromatic C=C stretch
1460, 1450		N-H bending
1260, 1248, 1225	S	N-O stretch, Aromatic C-N stretch

M=Medium; S=Strong

Other characteristic bands appear at 1032, 989, 886, 775 and 768  $\text{cm}^{-1}$ . Similar results have been previously reported (24).

#### 4.6.4. Nuclear Magnetic Resonance (NMR) Spectra

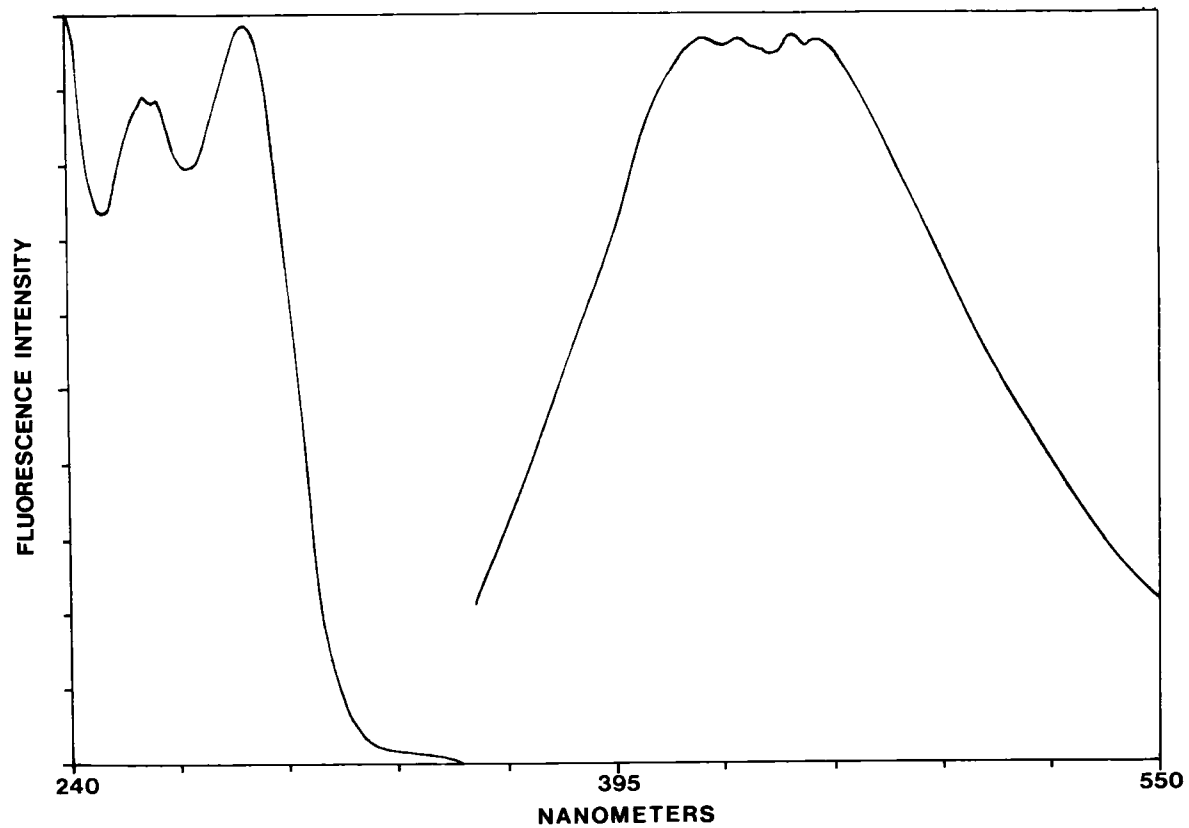


Figure 3. Excitation and Emission Fluorescence (corrected) Spectra of Minoxidil in Water.

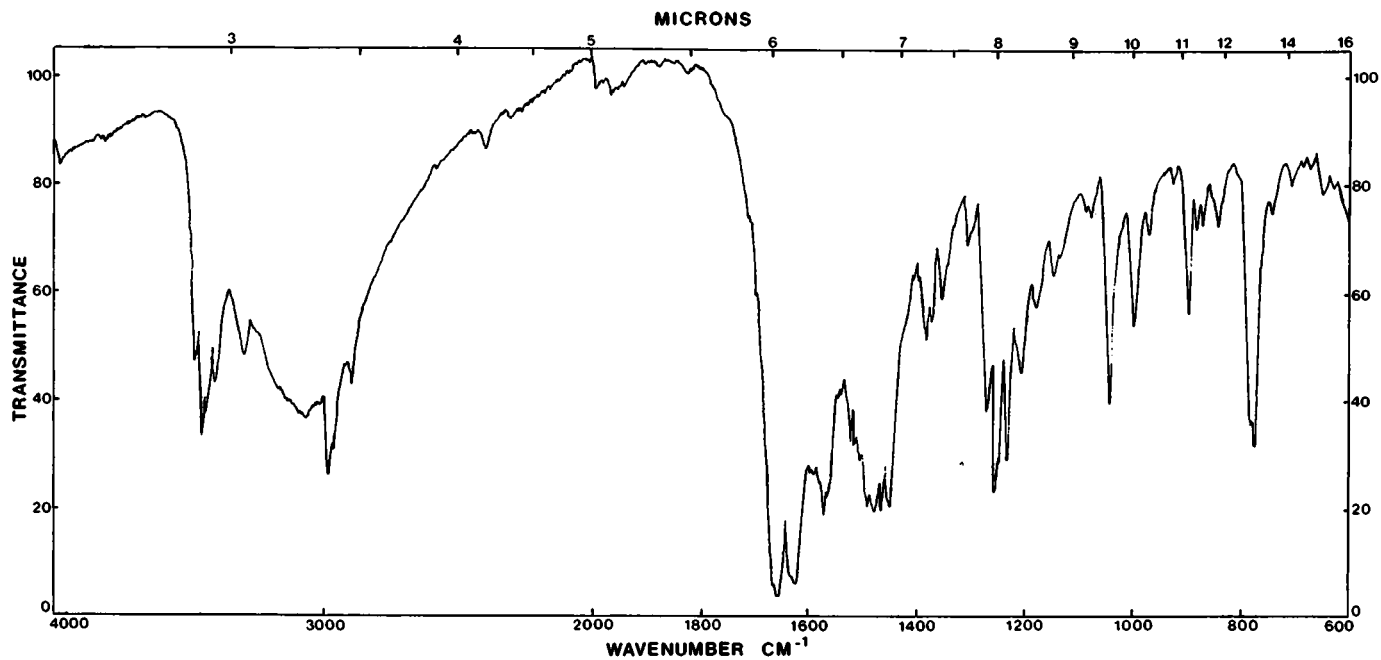
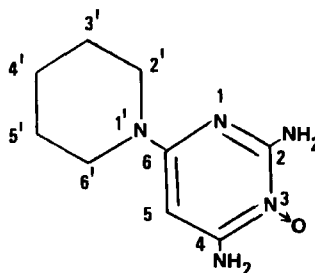


Figure 4. Infrared Spectrum of Minoxidil-KBr Disc.

#### 4.6.4.1. Proton Magnetic Resonance (PMR) Spectrum

The PMR spectrum of minoxidil in DMSO-d<sub>6</sub> is shown in Figure 5. The spectrum was recorded at ambient temperature on a Bruker AM 300 NMR spectrometer employing a frequency of 300.13 MHz. The chemical shifts, multiplicities and assignments are given in Table 4.

Table 4. Proton Magnetic Resonance Assignments for Minoxidil



Chemical Shift $\delta$ (ppm)	Multiplicities	Number of Protons	Assignment
1.48	m	4	3', 5'
1.55	m	2	4'
3.38	s, m	4	H <sub>2</sub> O, 2', 6'
5.36	s	1	5
6.80	s	4	NH <sub>2</sub> , 2, 4

M=Multiplet; S=Singlet

The PMR spectrum of minoxidil in DMSO-d<sub>6</sub> recorded at 70°C is given in Figure 6. The elevated temperature shifts the large HDO signal at 3.38 ppm upfield and subsequently reveals a triplet at 3.37 ppm which corresponds to the four 2' 6' protons. Moreover, the addition of deuterium oxide shifts the HDO signal downfield, uncovers the multiplet at 3.37 ppm corresponding to the 2' 6' protons and leads to the virtual disappearance of the NH<sub>2</sub> signal at 6.80 ppm (Figure 7).

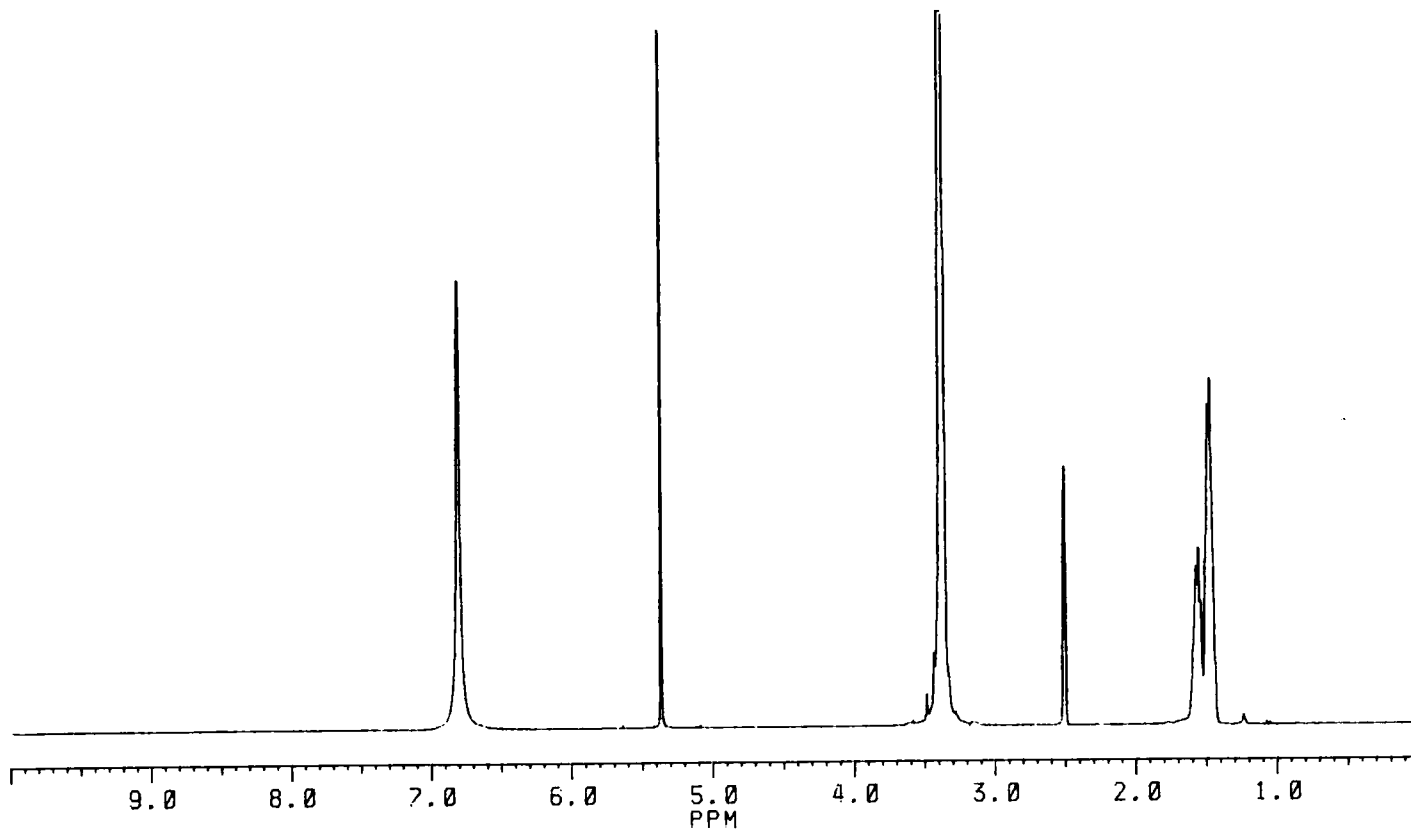


Figure 5. Proton Magnetic Resonance Spectrum of Minoxidil in DMSO-d<sub>6</sub>.

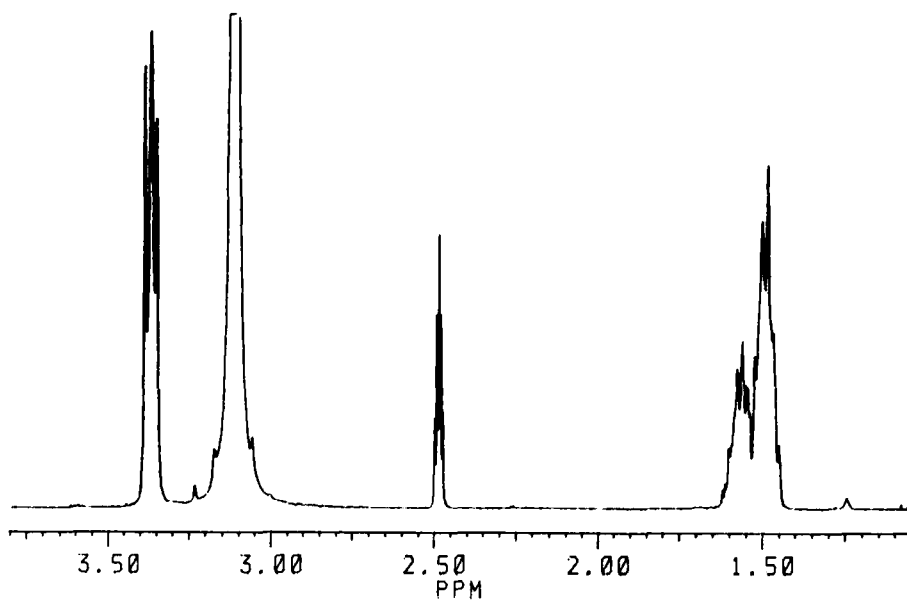


Figure 6. Proton Magnetic Resonance Spectrum of Minoxidil in DMSO-d<sub>6</sub> (recorded at 70°C); 1.25-3.75 ppm.

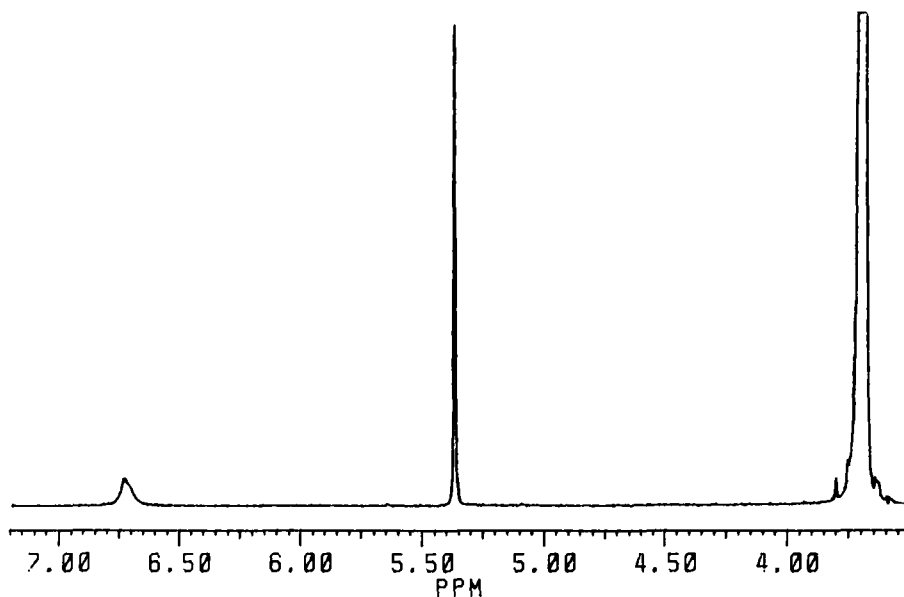


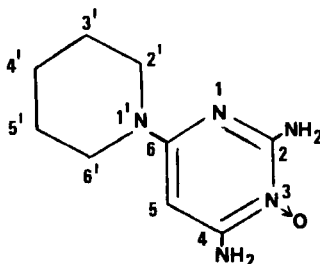
Figure 7. Proton Magnetic Resonance Spectrum of Minoxidil in DMSO-d<sub>6</sub> (D<sub>2</sub>O added); 3.50-7.00 ppm.



#### 4.6.4.2. Carbon-13 Nuclear Magnetic Resonance Spectrum ( $^{13}\text{C}$ -NMR)

The  $^{13}\text{C}$ -NMR spectrum of minoxidil obtained in  $\text{DMSO-d}_6$  is given in Figure 8. The spectrum was recorded at ambient temperature on a Bruker AM 300 NMR spectrometer at 75.47 MHz with broad band proton decoupling. Chemical shifts and structural assignments are outlined in Table 5.

Table 5. Carbon-13 Nuclear Magnetic Resonance Assignments for Minoxidil



Chemical Shift $\delta$ (ppm)	Assignment
24.03	4'
24.79	3', 5'
44.85	2', 6'
73.23	5
151.70	2
152.87	4
153.87	6

#### 4.6.5. Mass Spectrometry

##### 4.6.5.1. Electron Impact (EI)

The electron impact mass spectrum of minoxidil is shown in Figure 9. The spectrum was obtained by direct solid insertion probe at an electron energy of 70 eV and a source temperature of  $180^\circ\text{C}$  using a VG Analytical 70-70 HS double focussing mass spectrometer linked to a VG 2035 data system. The spectrum shows a molecular ion

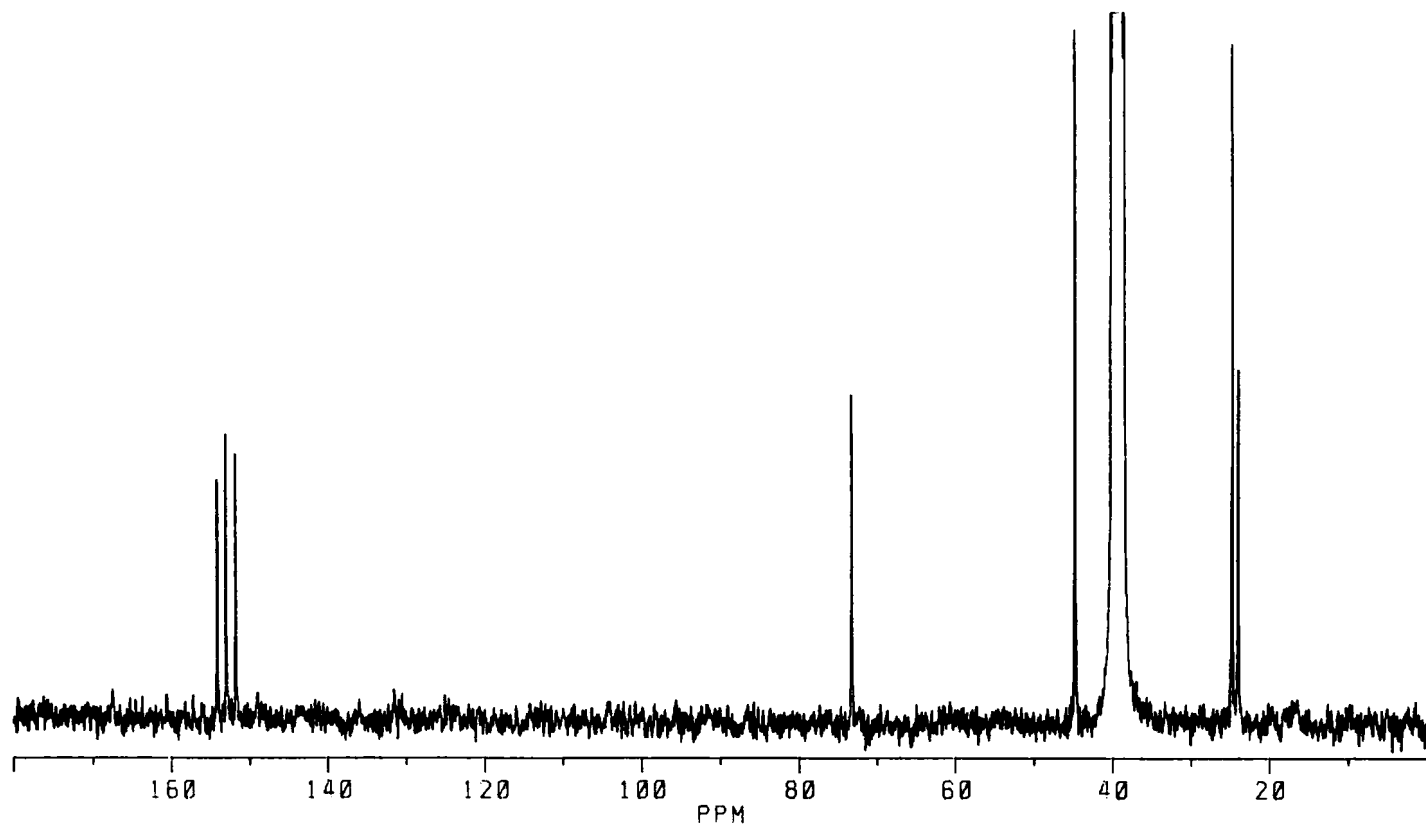


Figure 8. Carbon-13 Nuclear Magnetic Resonance Spectrum of Minoxidil in DMSO-d<sub>6</sub>.

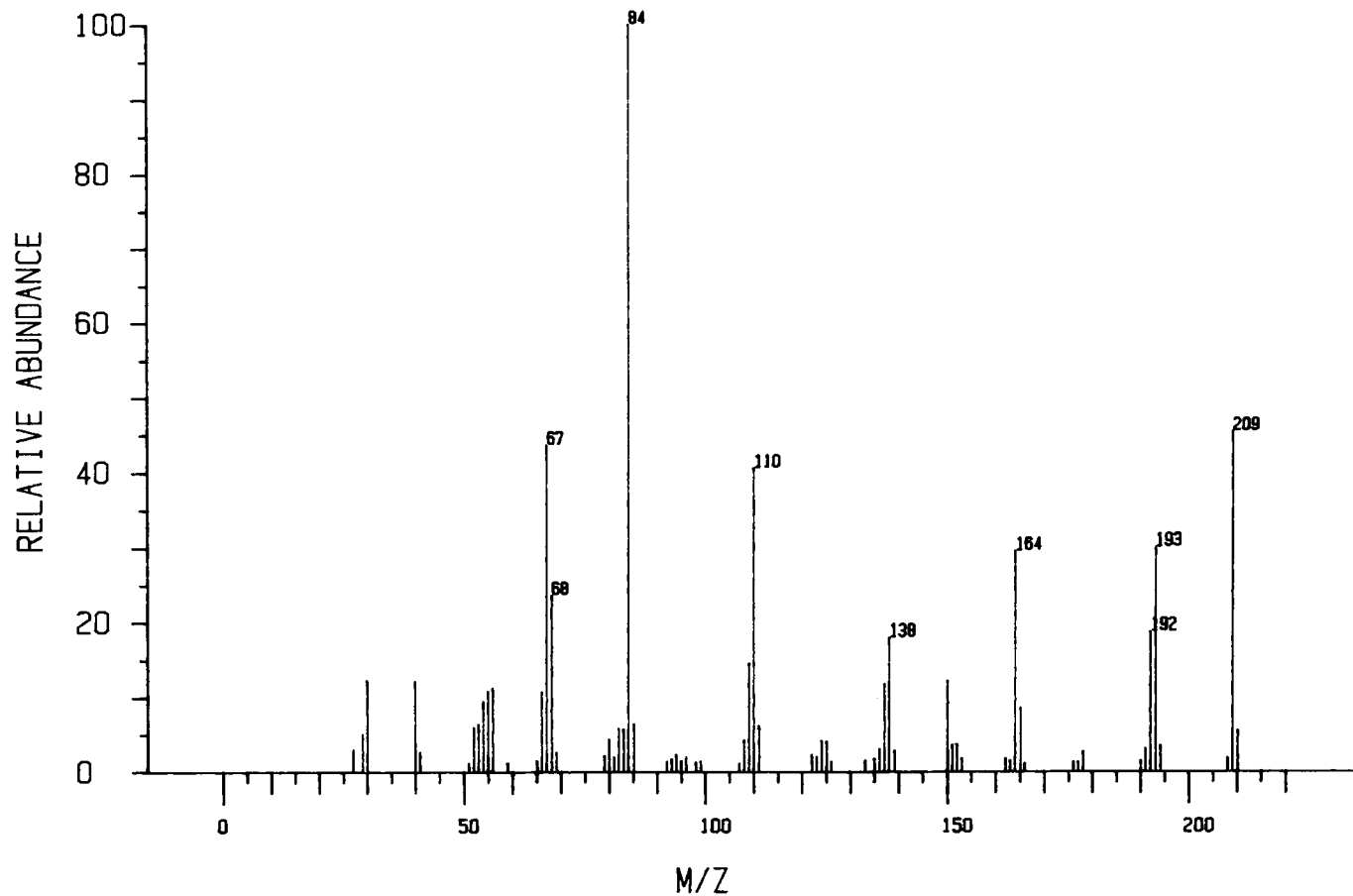


Figure 9. Mass Spectrum of Minoxidil-Electron Impact.

peak  $M^+$  at a mass/charge ( $m/z$ ) ratio of 209 with a relative intensity of 45.4% and a base peak at 84. The most prominent ions, their relative intensities and corresponding proposed fragment ions are presented in Table 6.

The mass spectrum of minoxidil was previously reported (24) with fragment ions of 84, 209, 67, 43, 110, 41, 192 and 164 in descending order of relative intensity.

#### 4.6.5.2. Chemical Ionization (CI)

The chemical ionization spectrum of minoxidil is presented in Figure 10. This spectrum was obtained on a VG Analytical 70-70 HS double focussing mass spectrometer equipped with a VG 2035 data acquisition system. Ammonia was used as the ionizing gas. The spectrum displays a pseudo molecular ion at a mass / charge ratio of 210 and a fragment ion at 194 which corresponds to the loss of O from  $MH^+$ .

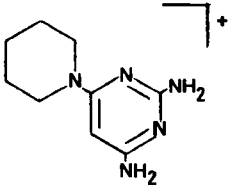
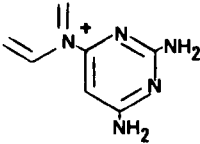
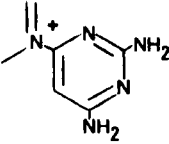
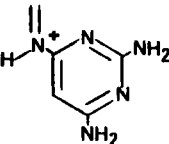
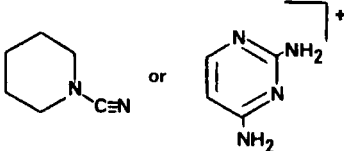
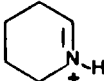
#### 4.6.5.3. Fast Atom Bombardment (FAB)

The positive ion fast atom bombardment spectrum of minoxidil is given in Figure 11. The spectrum was obtained on a VG Analytical 70SQ double focussing hybrid mass spectrometer coupled to a VG 11-250J data system. Approximately 1  $\mu$ g of sample suspended in a glycerol matrix was applied to the FAB target. The data was recorded at ambient temperature using argon as a source of fast atoms delivered with an energy of 8 kV at 1 mA current. A pseudo molecular ion,  $[MH^+]$ , is observed at  $m/z$  210 with the base peak and a  $[2MH]^+$  ion at  $m/z$  194 and  $m/z$  419 respectively.

## 5. Synthesis

The major synthetic routes used in the preparation of minoxidil are presented in Scheme 1. The methods I-VIII and IV-V-VIII are routinely employed in the manufacturing of raw material for pharmaceuticals whereas method IV-VI-VII-VIII is

Table 6. Mass Spectral (EI) Data for Minoxidil

$m/z$	Relative Intensity (%)	Fragment Ion
209	45.4	$M^+$
193	30.1	
164	29.6	
150	12.2	
138	18	
110	40.6	
84	100	

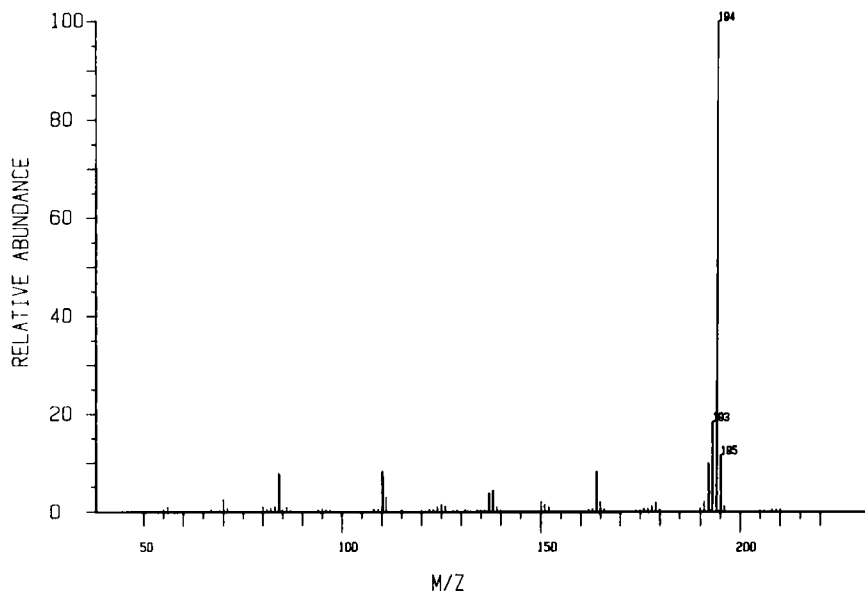


Figure 10. Mass Spectrum of Minoxidil-Chemical Ionization.

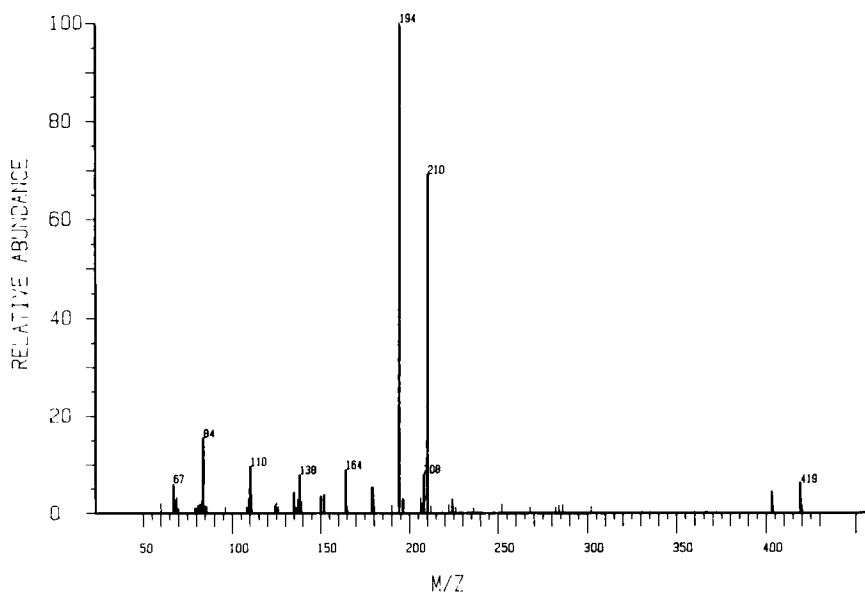
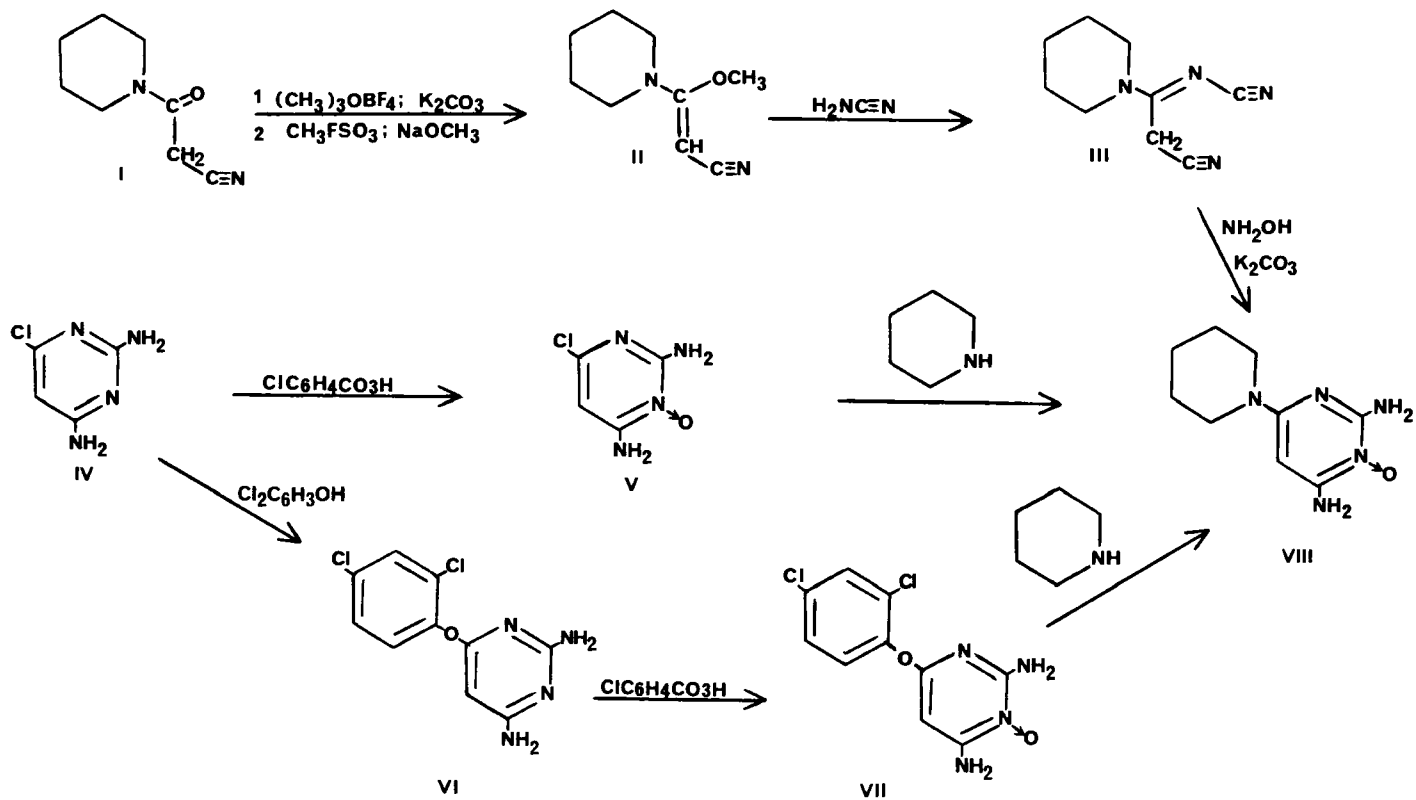


Figure 11. Mass Spectrum of Minoxidil-Fast Atom Bombardment.



Scheme 1. Synthesis of minoxidil

described in an original patent on 1,2-dihydro-1-hydroxy-pyrimidines (4). The synthetic route IV-V-VIII has also been employed in the preparation of  $^{14}\text{C}$ -labeled minoxidil (32).

Minoxidil, a piperidinopyrimidine N-oxide, can be prepared from either substituted piperidine or pyrimidine starting materials. A novel approach utilizes a piperidine derivative which eventually undergoes a cyclization reaction to simultaneously form both the diaminopyrimidine ring and N-oxide function (31). Reacting N-(2-cyanoacetyl)piperidine (I) with either trimethyloxonium fluoroborate or methylfluorosulfonate yields the enol ether (II) which when treated with cyanamide gives the cyanoiminopropionitrile (III). Subsequent reaction with hydroxylamine and potassium carbonate affords the desired pyrimidine N-oxide (VIII).

6-Chloro-2,4-diaminopyrimidine (IV) is a common starting material in other synthetic routes. Reaction with *m*-chloroperbenzoic acid gives the N-oxide (V) which when treated with piperidine yields minoxidil. Alternatively, condensation with 2,4-dichlorophenol, followed by perbenzoic acid oxidation and subsequent reaction with piperidine also produces minoxidil.

A number of variations of the above described methods have recently been reported (20-23).

## 6. Pharmacokinetics

### 6.1. Absorption

Minoxidil is rapidly and almost completely absorbed (95%) after oral administration and usually appears in the blood within 30 minutes. Peak plasma levels are reached within 1 hour of administration, however, peak concentrations that are reported vary considerably (17,33-37). Cutaneous absorption of topically applied minoxidil is limited to less than 5% of the applied dose (35,36,38).

### 6.2. Distribution

The volume of distribution of minoxidil is in excess of total body water suggesting that it concentrates in extravascular sites. This was indeed confirmed with whole-body autoradiography studies in rats (32). Despite using different methods of analysis, similar volume of distribution values (approximately 2.8-3.7L/kg) were calculated for minoxidil after oral administration to



hypertensive patients (17,33). In vitro protein binding experiments (equilibrium dialysis) show that minoxidil is not significantly bound to plasma proteins (33). No reports describing transfer across the placenta or distribution into breast milk are available.

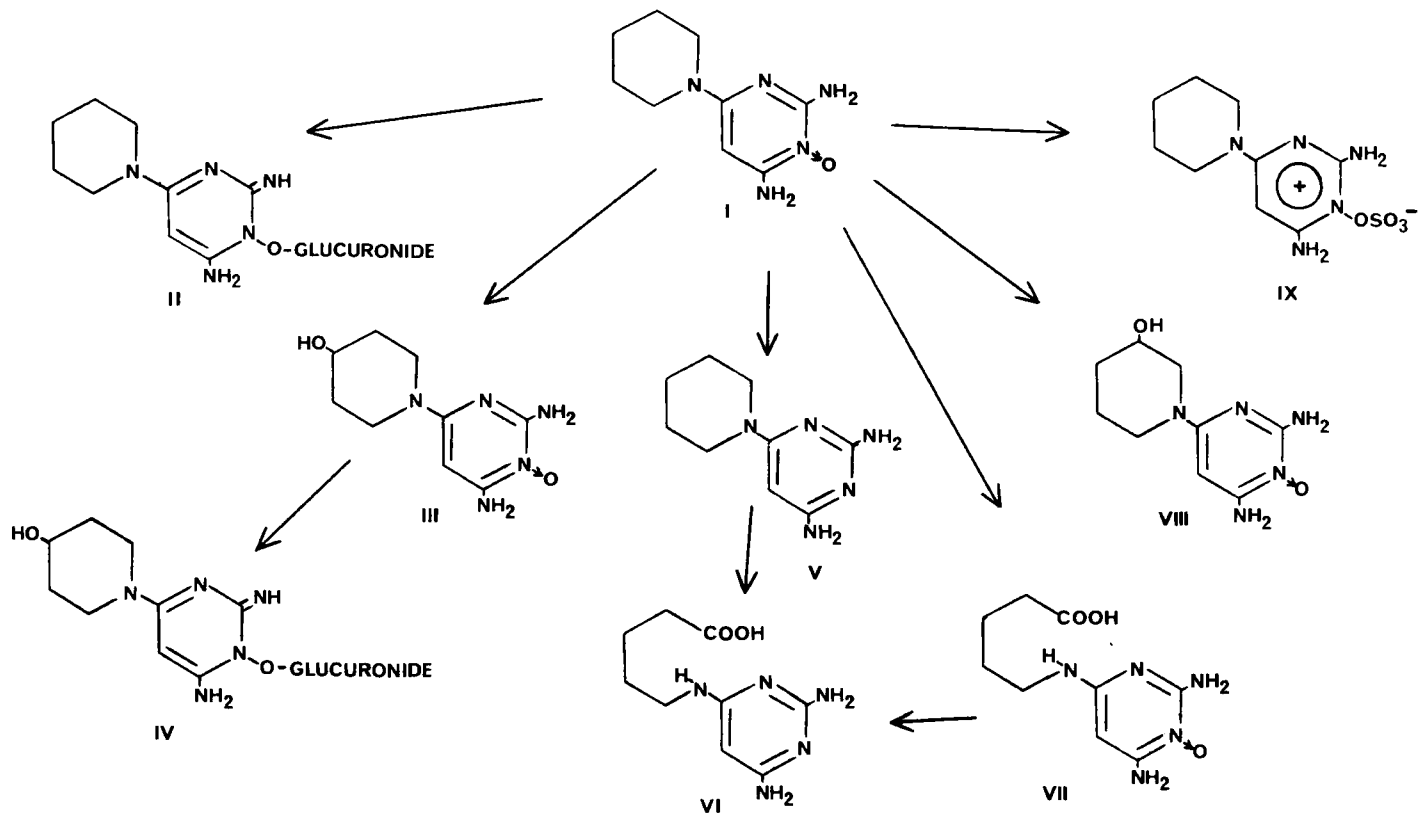
### 6.3. Elimination

Early data on the elimination half-life of minoxidil is unclear. One study, employing  $^{14}\text{C}$ -labeled drug in hypertensive individuals with varying degrees of renal impairment reported a serum half-life of 4.2 hours (33). When minoxidil was measured by radioimmunoassay in a similar group of patients elimination half-life values of 2.77 hours (tablet) and 3.09 hours (solution) were calculated (34). However, recent investigations suggest that the drug is eliminated more rapidly and that the half-life data reported is, at least in part, based on the assay method used. When the more specific methods were employed (high performance liquid chromatography, radioimmunoassay) a serum half-life ranging from 1.4 to 1.8 hours was obtained (37,38).

The biotransformation of minoxidil has been investigated in four species: man (33,34), rat, dog and monkey (32,39). The metabolites identified to date are presented in Scheme 2. In man, minoxidil (I) is primarily metabolized by conjugation with glucuronic acid at the N-oxide position to form minoxidil-0-glucuronide (II), (48-67%) (17,39). Others identified in man include: 2,4-diamino-6-(4'-hydroxypiperidino)pyrimidine 3-oxide (III) (4'-hydroxyminoxidil), 2,4-diamino-6-piperidinopyrimidine (IV) (reduced minoxidil) as well as minoxidil (I) itself (39).

All metabolites given in Scheme 2 have been identified in rat, dog and monkey with two exceptions. The glucuronide conjugate of 4'-hydroxyminoxidil (IV) has only been detected in dog (39) whereas minoxidil 0-sulfate (IX) has only been reported in rat (40). The latter compound, minoxidil 0-sulfate (IX), has recently gained considerable attention as a result of evidence suggesting a metabolic conversion of minoxidil to an active metabolite (41), and the studies showing that the sulfate is both a more potent hypotensive and direct-acting vasodilator (42). Its role as a hair growth stimulant is also being investigated (43).

In all the species studied, minoxidil and its metabolites are primarily excreted by the kidney. Greater than 90% of the administered drug ultimately



Scheme 2. Biotransformation pathways of minoxidil

appears in the urine whereas only 1-3% is found in the feces (17,32,39). The drug is excreted by glomerular filtration with no evidence of secretion or reabsorption from the renal tubules.

## 7. Methods of Analysis

### 7.1. Determination in Pharmaceuticals

#### 7.1.1. Spectrophotometric Methods

##### 7.1.1.1. Colorimetric

Minoxidil reacts with 1% copper (II) nitrate,  $(\text{Cu}(\text{NO}_3)_2)$ , reagent to form a stable green complex which displays an absorption maximum at 368 nm (44). The complex forms rapidly and is useful for the determination of minoxidil in bulk powder and tablet form. The procedure reported involves extraction of the drug from the dosage form, clarification, addition of the reagent, volume adjustment and spectrophotometric measurement.

##### 7.1.1.2. Ultraviolet

The United States Pharmacopeia - National Formulary (U.S.P.-N.F.) employs ultraviolet spectroscopy for the determination of minoxidil in samples resulting from dissolution testing of tablets containing the drug (45). For tablets containing up to 10 mg of the drug, measurement is made at 231 nm whereas those containing more than 10 mg, the absorption maximum at 287 nm is employed. Quantitation is based on the net absorbances of the amount dissolved as compared to that of a standard solution.

##### 7.1.1.3. Infrared

The U.S.P.-N.F. uses infrared spectroscopy to establish the identity of minoxidil in tablets containing the drug

(45). The infrared absorption spectrum of a 0.033% dispersion in potassium bromide is compared to that of a similar preparation containing the official reference standard.

#### 7.1.2. Chromatographic Methods

##### 7.1.2.1. Gas-Liquid Chromatography (GLC)

Gas chromatography has been used in the identification and quantitation of minoxidil in both tablets and bulk powder (46). The procedure described employs a packed column containing 1.5% OV-17 on Gas Chrom Q (80-100).

##### 7.1.2.2. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) has become the most important method for the determination of minoxidil in dosage forms. The technique offers obvious advantages over others as it is useful in the presence of possible decomposition products and any contaminating synthetic intermediates.

A reversed-phase ion-pairing method, recently developed (26), has found widespread application for bulk drug substance as well as tablet and solution forms of the drug. In addition, it has been adopted as the official method for assaying minoxidil powder and tablets (45). General assay conditions are as follows: i) isocratic conditions with ultraviolet detection (254 nm); ii) column, octadecylsilane (C<sub>18</sub>), 10 µm particles; iii) mobile phase, methanol:water:glacial acetic acid (700:300:10), pH 3.0 and containing sodium dioctylsulfosuccinate (3.0 g); iv) operating conditions, ambient temperature, flow rate, 1.0 mL/min, injection volume, 10 µL; v) sample preparation, standards, bulk powder, tablet and solution samples (0.25 mg/mL), internal standard, medroxyprogesterone acetate (0.20 mg/mL).

A general HPLC method developed for the routine analysis of a wide range of unrelated drugs in typical dosage forms is also applicable to minoxidil (47).

Chromatographic conditions include: i) ultraviolet detection (280 nm); ii) column, octadecylsilane (C<sub>18</sub>), 10  $\mu$ m particles; iii) mobile phase, acetonitrile (15%) in 0.05 M potassium dihydrogen phosphate; iv) operating conditions, ambient temperature, flow rate, 2.0 mL/min., injection volume, 20  $\mu$ L; v) sample preparation, tablet (0.10 mg/mL).

### 7.1.3. Miscellaneous Methods

#### 7.1.3.1. Capillary Isotachophoresis

Capillary isotachophoresis has been successfully employed in the determination of minoxidil in tablet and solution forms (48). Analysis was performed with an isotachophoretic analyzer equipped with a PTFE capillary tube (230 cm x 0.5 mm I.D.). The initially applied current, 130  $\mu$ A, was reduced during analyses and switched off at 10  $\mu$ A for detection. Potassium acetate-acetic acid buffer (pH 5.1) (10 mM) and  $\beta$ -alanine (10 mM) were the preferred leading and terminating electrolytes respectively although the detection limit increased with 2-pyridinecarboxylic acid as the leading electrolyte.

#### 7.1.3.1. Differential-Pulse Polarography

The electrochemical reduction of the N-oxide bond of the minoxidil molecule forms the basis of a differential-pulse polarographic assay of the drug in tablets (49). A single methanol extraction followed by electroreduction in H<sub>2</sub>SO<sub>4</sub> results in an intense, well resolved differential-pulse wave, E<sub>1/2</sub> (half-wave potential) value of -0.95 V, which is ideal for drug quantitation (relating peak amplitude to concentration). The procedure developed is useful for both tablet content uniformity and general tablet assay.

A coulometric analysis of minoxidil revealed that four electrons per molecule were involved in the electroreduction process. The initial peak potential ( $E_{\frac{1}{2}} = -0.95$  V, 2 electrons) corresponds to the reduction of the protonated N-oxide bond therefore forming 2,4-diamino-6- piperidinopyrimidine. A second 2-electron process ( $E_{\frac{1}{2}} = -1.20$  V) involving reduction of the 3,4 carbon-nitrogen (azomethine) double bond with subsequent deamination was confirmed by the isolation of 2-amino-6-piperidinopyrimidine (49).

## 7.2. Determination in Biological Fluids

### 7.2.1. High Performance Liquid Chromatography

A number of HPLC methods have been developed which are suitable for the measurement of minoxidil and/or minoxidil sulfate in biological fluids (37,40,41,51,52). All methods employ reverse-phase or reversed-phase ion-pairing chromatography utilizing either octylsilyl ( $C_8$ ) or octadecylsilyl ( $C_{18}$ ) column packing and an aqueous-organic modifier mobile phase. Detector systems include ultraviolet (UV) monitored at 280 nm or electrochemical oxidation. A summary of these HPLC systems is given in Table 7.

### 7.2.2. Radioimmunoassay (RIA)

A sensitive and specific radioimmunoassay for minoxidil in human serum has been described (38). Antiserum, produced by immunizing rabbits with the bovine serum albumin conjugate of the N-4-glutaryl derivative, showed little cross-reactivity with the drugs metabolites. The procedure employing  $^3H$ -labeled minoxidil, requires relatively long equilibration times, however, small sample volumes (50  $\mu$ L or less) and minimal sample preparation. A limit of detection of 3.02 ng/mL could be improved upon by further primary antibody dilution and larger sample volumes. The usefulness of the RIA procedure in pharmacokinetic studies was demonstrated by monitoring human serum levels for up to 24 hours following a single oral dose of the drug.

Table 7. HPLC Systems for Minoxidil in Biological Fluids.

<u>Sample</u>	<u>Column</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>Reference</u>
Plasma	$\mu$ Bondapak C <sub>18</sub> , 30cm x 3.9mm, 10 $\mu$ m	Isocratic; H <sub>2</sub> O: CH <sub>3</sub> CN (98:11), pH 3.0 (H <sub>3</sub> PO <sub>4</sub> )	Coulometric, sensitivity- 0.5 ng/mL	37
Platelet homo- genate incubation	LiChrosorb RP-8, 25cm x 4.6mm, 10 $\mu$ m	Isocratic; 1mL/L CF <sub>3</sub> COOH containing 30% CH <sub>3</sub> CN	UV, 280nm	51
Serum	Spherisorb C <sub>8</sub> , 25cm x 2.1mm, 5 $\mu$ m	Isocratic; H <sub>2</sub> O:CH <sub>3</sub> CN: THF (70:25:5) with 0.01M sodium n-octane- sulphate, ammonium citrate and 0.03M HClO <sub>4</sub> pH 3.2 (NH <sub>4</sub> OH)	Amperometric, sensitivity - 0.3 ng/mL	52
Bile	LiChrosorb RP-8, 25cm x 4.6mm, 10 $\mu$ m	Gradient; A: 0.01MNaH <sub>2</sub> PO <sub>4</sub> (pH 2.8): CH <sub>3</sub> CN(95:5) B: 0.01MNaH <sub>2</sub> PO <sub>4</sub> (pH 2.8): CH <sub>3</sub> CN(50:50)	UV, 280 nm	40
Liver homogenate incubation	LiChrosorb RP-8 25cm x 4.6mm, 10 $\mu$ m	Gradient; 0.01M NaH <sub>2</sub> PO <sub>4</sub> (pH 2.8): CH <sub>3</sub> CN (85:15)	UV, 280 nm	41

### 7.3. General - Thin-Layer Chromatography (TLC)

Three thin-layer chromatography (TLC) systems developed for use in a general screening method for nitrogenous basic drugs may be used as an identification guide for minoxidil (50). TLC plates of silica gel G (250  $\mu\text{m}$  thick) are sprayed with or dipped in 0.1 M potassium hydroxide in methanol and then dried. A recommended mobile phase and its corresponding  $\text{rf}$  value for minoxidil are as follows: i) methanol:strong ammonia solution (100:1.5),  $\text{rf}$  51; ii) cyclohexane:toluene:diethylamine (75:15:10),  $\text{rf}$  00; iii) chloroform:methanol (90:10),  $\text{rf}$  03 (24).

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# MITOXANTRONE HYDROCHLORIDE

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## 1. INTRODUCTION

The proven clinical usefulness of doxorubicin in current chemotherapy practice has stimulated much research to discover new agents with similar modes of action but without the dose-limiting cardiotoxicity of the class of anthracyclines (1-5). From extensive structure-activity studies it appeared that a planar quinoid ring system, potentially capable of DNA intercalation, linked with a basic amino function in a side chain for stabilization of the intercalated complex, are essential structural features for binding to DNA (6,7). Furthermore it was hypothesized that the cardiotoxicity of the anthracyclines doxorubicin and daunorubicin is in some manner related to the daunosamine sugar moiety (8). Replacement of the daunosamine sugar moiety by an alkylamino-substituted side chain should eliminate the untoward cardiac effects (8-11). As a result of these efforts the aminoanthracenedione derivative, mitoxantrone hydrochloride (Figure 1), was synthesized (1,2,12,13). The drug has shown antitumour activity in several *in vitro* systems, experimental animal models and in humans (14-35). Clinical studies indicate that mitoxantrone is at least as effective as doxorubicin in breast cancer but with apparently reduced cardiotoxicity. This makes the drug an important addition to the chemotherapy armamentarium of today's oncology practice.

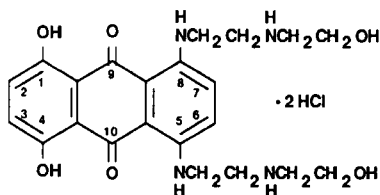


Figure 1  
Structure of mitoxantrone hydrochloride

## 2. DESCRIPTION

### 2.1 Name, Formula, Molecular Weight

The generic name is mitoxantrone hydrochloride. The Chemical Abstracts name is 1,4-dihydroxy-5,8-bis((2-((2-hydroxyethyl)amino)ethyl)amino)-9,10-anthracenedione dihydrochloride. Other names, abbreviations and drug code numbers are:

DHAD, DHAQ, CL 232315, NSC-301739 (36). Mitoxantrone hydrochloride is marketed by Lederle Laboratories/American Cyanamid Company, Pearl River, New York, USA under the trademark of Novantrone®. The empirical molecular formula for mitoxantrone hydrochloride is  $C_{22}H_{28}N_4O_6 \cdot 2HCl$ . The molecular weight is 517.41.

## 2.2 Appearance, Colour, Odour

The hydrochloride salt is a dark, blue-black, free-flowing crystalline powder without odour. The compound is hygroscopic. At relative humidities of 30 to 40% the compound appears to have formed a trihydrate whereas at relative humidities from 50 to 80% the percentage of moisture is what would be expected for a tetrahydrate (37).

## 3. SYNTHESIS

Synthetic pathways have been described for the preparation of mitoxantrone hydrochloride (1,2,12,13). The synthetic route, published by Murdock et al.(1), is presented in Figure 2.

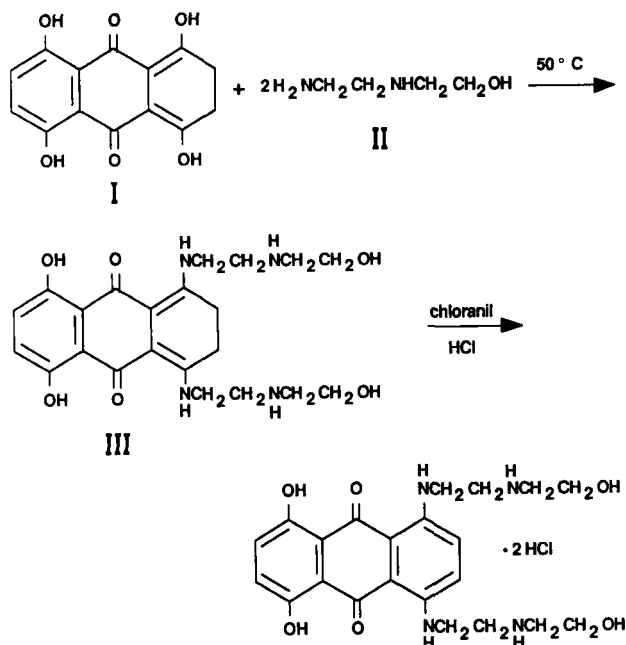


Figure 2  
Chemical synthesis of mitoxantrone hydrochloride

The starting material, leuco-1,4,5,8-tetrahydroxyanthraquinone (I), reacts with 2-((2-amino-ethyl)amino)ethanol (II) to form 1,4-dihydroxy-6,7-dihydro-5,8-bis((2-((2-hydroxy-ethyl)amino)ethyl)amino)-9,10-anthracenedione (III). The product III is aromatized, by using chloranil as the oxidant, and it is converted into mitoxantrone hydrochloride under treatment with hydrogen chloride in ethanol. The crude product can be recrystallized from water-ethanol mixture. Tritiated mitoxantrone of high specific radioactivity (15 Ci/mmol) has been synthesized to be used as a radio-tracer ligand in a radioimmunoassay for the drug in serum samples (38).  $^3\text{H}$ -mitoxantrone was prepared from unlabeled compound by catalytic tritium exchange.

#### 4. PHYSICAL PROPERTIES

##### 4.1 Ultraviolet-Visible Spectrum

The ultraviolet-visible spectra of mitoxantrone hydrochloride ( $1.3 \times 10^{-5} \text{ M}$ ) in 0.05 M HCl (Spectrum 1) and in 0.05 M NaOH (Spectrum 2) are depicted in Figure 3. The spectra were recorded by using a Pye Unicam SP8-400 UV-VIS double beam spectrophotometer and 1 cm quartz cells. Reported UV-VIS spectral data, in terms of absorption maxima ( $\lambda_{\text{max}}$ ) and molar absorptivities ( $\epsilon$ ), for mitoxantrone hydrochloride in water have been listed in Table I.

Table I                      UV-VIS Spectral Data for Mitoxantrone Hydrochloride in Water (1)

$\lambda_{\text{max}}$ (nm)	$\epsilon$ ( $\text{l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ )
241	41,000
273	12,000
608	19,200
658	20,900

Molar extinction coefficients for mitoxantrone hydrochloride at 662, 611 and 246 nm in the chromatographic eluent 4.4 M ammonium formate buffer (pH 4.3) + acetonitrile + water in a ratio of 2:1:1 are:  $24 \times 10^3$ ,  $19 \times 10^3$  and  $40 \times 10^3$ , respectively (39).



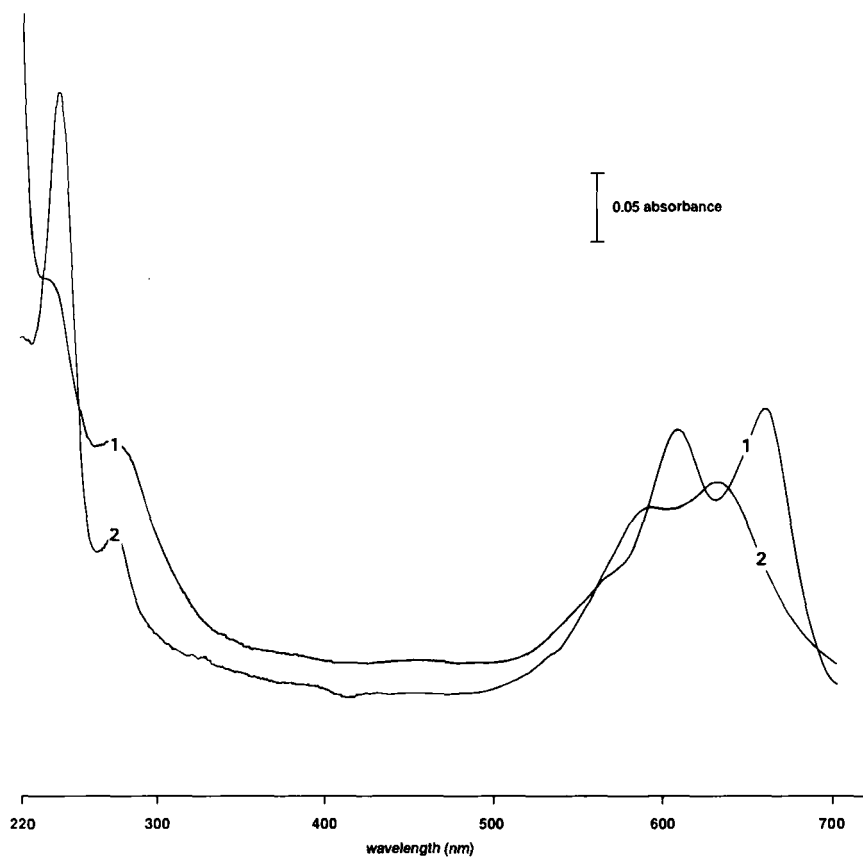


Figure 3

UV-VIS spectra of mitoxantrone hydrochloride in 0.05 M HCl (Spectrum 1) and in 0.05 M NaOH (Spectrum 2)

#### 4.2 Infrared Spectrum

The infrared spectrum of mitoxantrone hydrochloride (lot nr. 5823) is presented in Figure 4. The spectrum was recorded with a Jasco A100 infrared spectrophotometer from a compressed potassium bromide disc. Tentative structural assignments of some of the characteristic absorption bands are listed in Table II.

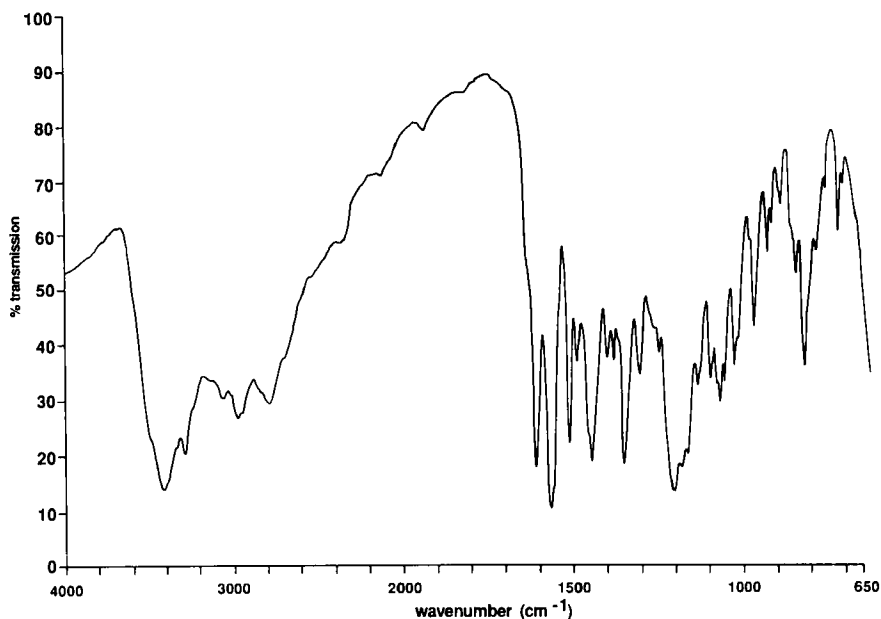


Figure 4  
Infrared spectrum of mitoxantrone hydrochloride

Table II              Infrared Assignments for Mitoxantrone Hydrochloride

wavenumber (cm <sup>-1</sup> )	assignment
2800-3400 <sup>s,br</sup>	$\nu(\text{OH})$ , $\nu(\text{NH})$ , $\nu(\text{NH}^+_2)$ and $\nu(\text{CH})$
1615 <sup>s</sup> , 1570 <sup>s</sup>	$\nu(\text{C}=\text{O})$ of quinone
1450 <sup>s</sup>	$\nu(\text{CH}_2)$
1210 <sup>s</sup>	$\nu(\text{C}-\text{O})$
825 <sup>s</sup>	p-disubstituted benzene

#### 4.3 Nuclear Magnetic Resonance (NMR) Spectrum

The proton NMR spectrum was recorded in D<sub>2</sub>O containing H<sub>2</sub>O as internal reference and using a Bruker WP-200 WB spectrometer at a frequency of 200.13 MHz (40). The spectrum

(detail) is presented in Figure 5 and refers to the situation after proton exchange. The spectral assignments are presented in Table III and correspond with the assignments published by Murdock et al. (1), while a further refinement is made (41).

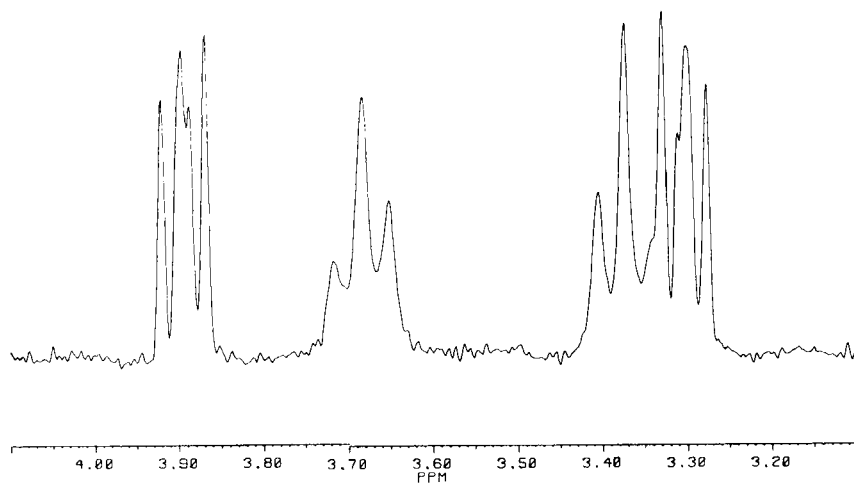
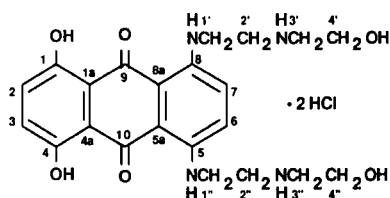


Figure 5  
Proton NMR spectrum of mitoxantrone hydrochloride (detail)

Table III  $^1\text{H}$ -NMR Assignment for Mitoxantrone Hydrochloride in  $\text{D}_2\text{O}$

Chemical shift $\delta$ (ppm)	Multiplicity	Number of atoms	Assignment
3.31	double doublet	4	$\text{H}_2\text{-3}', \text{-3}''$
3.38	triplet	4	$\text{H}_2\text{-2}', \text{-2}''$
3.69	triplet	4	$\text{H}_2\text{-1}', \text{-1}''$
3.90	double doublet	4	$\text{H}_2\text{-4}', \text{-4}''$
6.83	singlet	2	H-2, H-3
6.91	singlet	2	H-6, H-7

The natural abundance carbon 13 NMR spectrum was recorded on the same instrument at a frequency of 50.31 MHz (40). Acetone in  $\text{D}_2\text{O}$  was used as external reference (signal: 30.2 ppm). The proton-noise decoupled spectrum is presented in Figure 6 and the spectral assignments are summarized in Table IV (41).

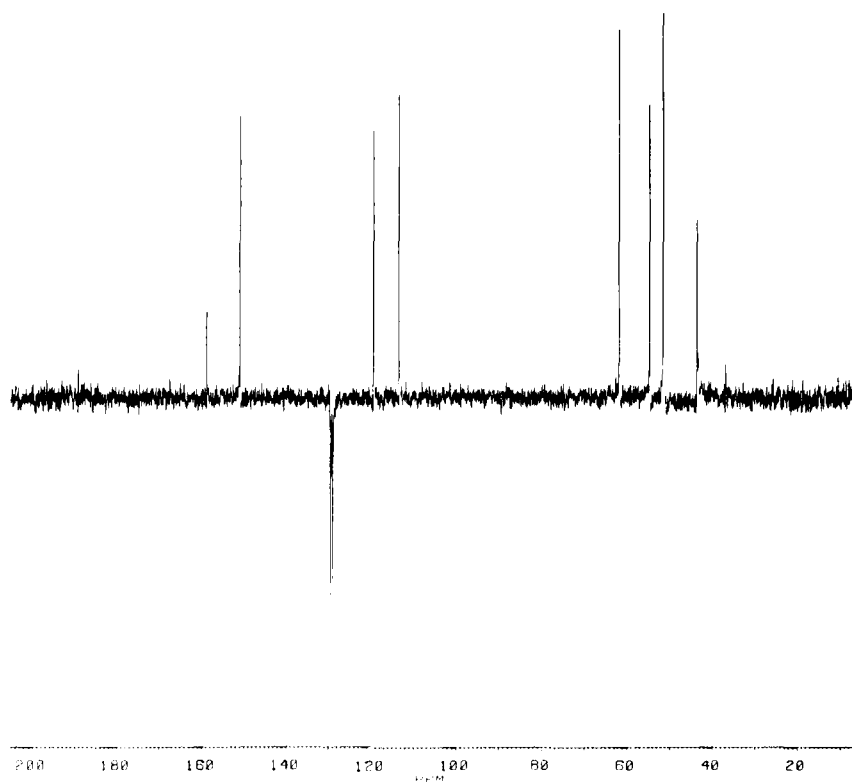


Figure 6  
Carbon 13 NMR spectrum of mitoxantrone hydrochloride

Table IV  $^{13}\text{C}$ -NMR Assignment for Mitoxantrone Hydrochloride in  $\text{D}_2\text{O}$

Chemical shift $\delta(\text{ppm})$	Assignment
43.5	C-3', C-3''
51.3	C-2', C-2''
54.5	C-1', C-1''
61.3	C-4', C-4''
113.3 <sup>a</sup>	C-5 <sup>a</sup> , C-8 <sup>a</sup>
119.4 <sup>a</sup>	C-1 <sup>a</sup> , C-4 <sup>a</sup>
128.9 <sup>b</sup>	C-6, C-7
129.4 <sup>b</sup>	C-2, C-3
150.8 <sup>c</sup>	C-5, C-8
158.7 <sup>c</sup>	C-1, C-4
188.9	C-9, C-10

a,b,c values are interchangeable

#### 4.4 Mass Spectrum

Mass spectral data for mitoxantrone hydrochloride are scanty. Therefore, for this analytical profile we recorded electron impact (EI), chemical ionization (CI), field desorption (FD) and fast atom bombardment (FAB) spectra of mitoxantrone hydrochloride (lot nr. 5823). CI and FAB mass spectroscopy were performed in the positive and negative ion mode. EI mass spectra were obtained by direct probe analysis on a Finnigan TSQ (MS-MS) mass spectrometer connected with an Incos data system. Conditions: electron energy, 70 eV; ion source temperature, 120°C; emission current, 0.20 mA. The molecular ion at  $m/z$  444 is not detectable in the EI mass spectrum. Direct insertion probe CI mass spectra were obtained by using a Finnigan TSQ (MS-MS) mass spectrometer coupled with the Incos data system. Methane gas was used as ionising reagent. The ion source pressure was 0.50 Torr, the ion source temperature was 120°C, the emission current was 0.20 mA and the multiplier voltage was 1800 V. FD mass spectra were obtained with a Varian MAT 711 double focussing mass spectrometer equipped with a MAT 100 data acquisition unit. 10  $\mu\text{m}$  Tungsten wire FD emitters containing carbon microneedles with an average length of 30  $\mu\text{m}$  were used. The sam-

ple was dissolved in methanol and then loaded onto the emitters by the dipping technique. An emission current of 12 mA was used to desorb the sample. The ion source temperature was 70°C. FAB mass spectrometry was carried out using a V.G. MICROMASS ZAB-2HF mass spectrometer, an instrument with reverse geometry, fitted with a high field magnet and coupled to a V.G. 11/250 data system. The samples were loaded in thioglycerol solution onto a stainless steel probe and bombarded with xenon atoms having a 8 keV energy (42,43). The recorded spectra are depicted in the Figures 7, 8, 9, 10, 11 and 12.

A high resolution mass spectrum exhibited the molecular ion at  $m/z$  444.2030 with composition  $C_{22}H_{28}N_4O_6$  (37).

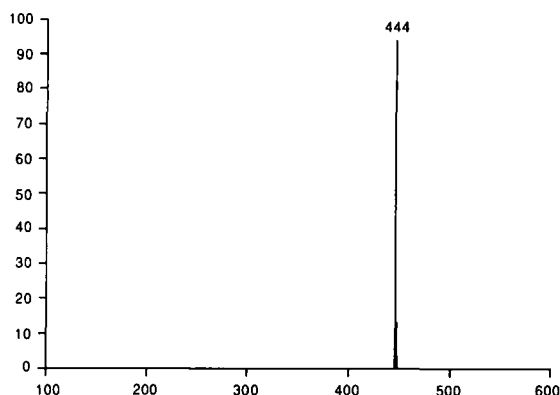
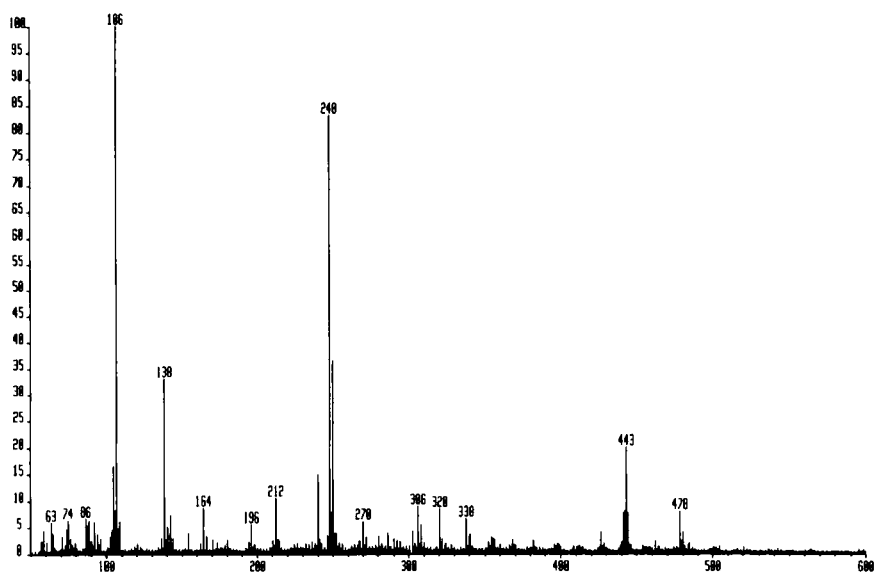
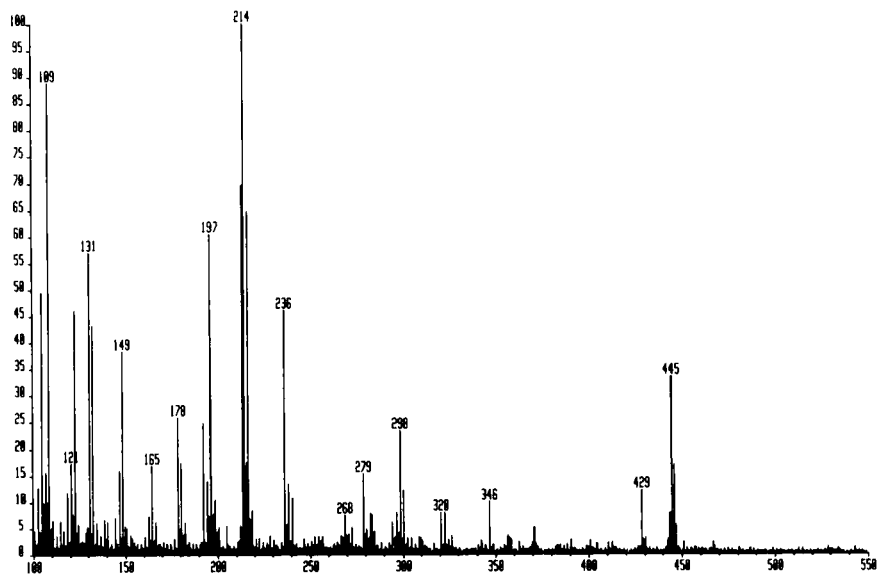


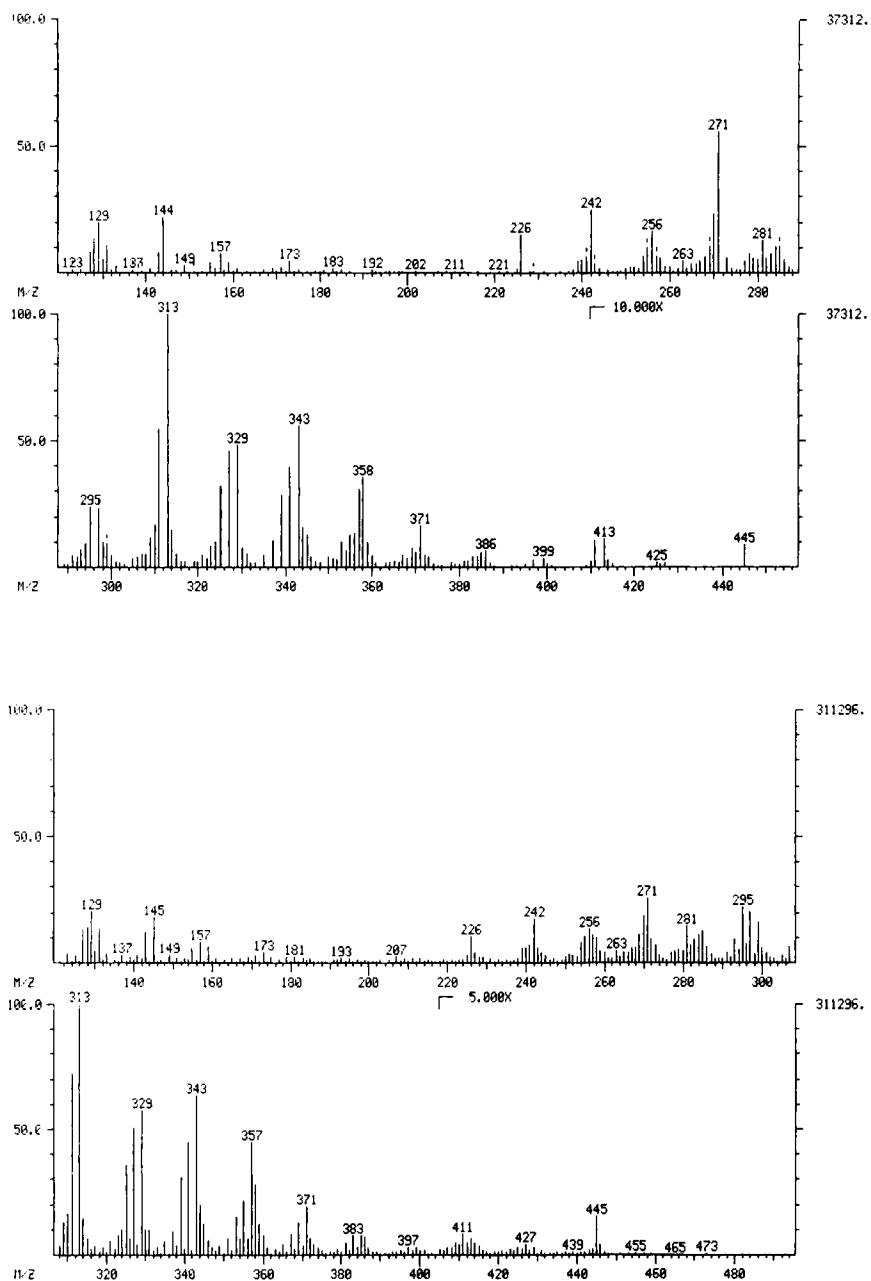
Figure 7  
FD mass spectrum of mitoxantrone hydrochloride



Figures 8/9

FAB (+) mass spectrum (upper spectrum) and FAB (-) mass spectrum (lower spectrum) of mitoxantrone hydrochloride





Figures 10/11

CI (+) mass spectrum (upper spectrum) and CI (-) mass spectrum (lower spectrum) of mitoxantrone hydrochloride

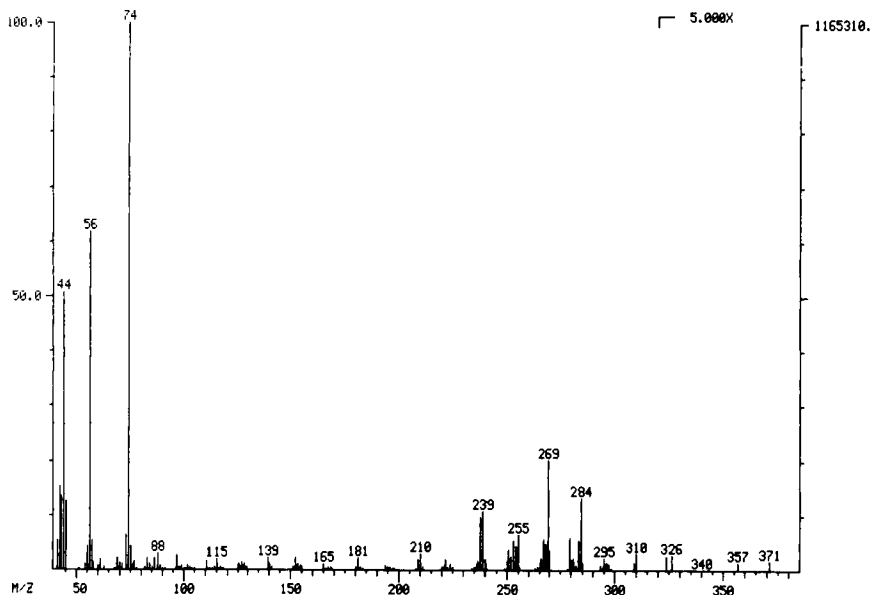


Figure 12

EI mass spectrum of mitoxantrone hydrochloride

#### 4.5 Optical Rotation/Circular Dichroism (CD) Spectrum

Due to the absence of chiral centers in the mitoxantrone molecule the drug exhibits no optical activity.

#### 4.6 Melting Point

The melting point for mitoxantrone base, recrystallized from a mixture of ethanol and hexane, is 160–162°C (2). The melting point for anhydrous mitoxantrone hydrochloride has been reported to be 203–205°C (uncorrected)(1). The melting points of lots PC 0463 and 5823 have been measured under European Pharmacopeia conditions. The result was in both cases 283°C. The samples were used as received. The determination of the melting range is hindered by decomposition of the sample before melting (37).

#### 4.7 Thermogravimetric Analysis

Thermogravimetric analysis of a sample of mitoxantrone hydrochloride revealed a weight loss of 5.6% at 150°C (37).

#### 4.8 Differential Scanning Calorimetry

The thermograms were recorded with a Seteram 111 or Perkin Elmer DSC-2 in air, the heating rate was 3 or 5 K/min while the sample size was about 3 mg (44). Samples of various origin were used (Lederle, lot nr. 0463 and 5823).

The thermograms of a specified sample of mitoxantrone hydrochloride are strongly variable in shape and measured temperature effects. The two more or less characteristic thermograms (temperature range 25 to 120°C) are presented in Figures 13 and 14. A rather sharp endothermic phase transition of about 80 J/gram is found around 73°C, sometimes a small endothermic pre-transition of 10 J/gram is seen at about 50°C. The total transition energy in the temperature range of 50 to 110°C varies strongly and can reach values up to 250 J/gram. The origin of this variation is uncertain.

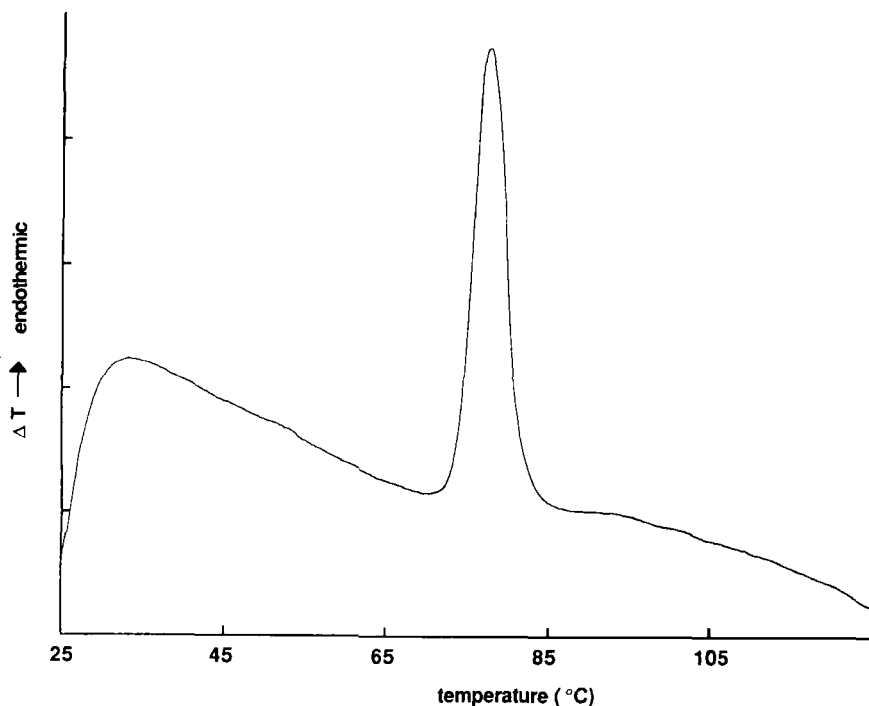


Figure 13  
DSC thermogram of mitoxantrone hydrochloride

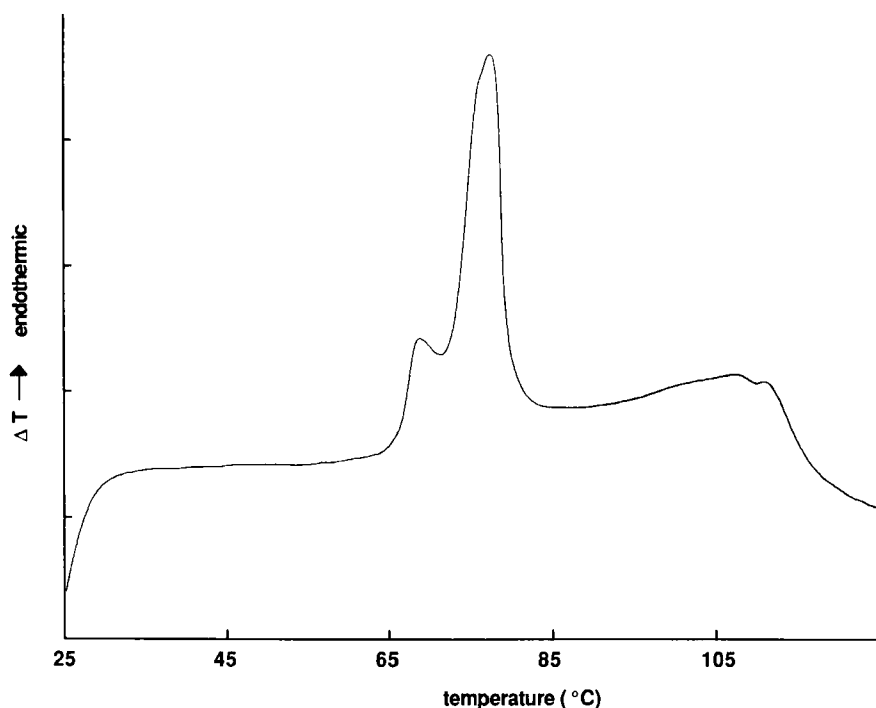


Figure 14  
DSC thermogram of mitoxantrone hydrochloride

Around 237°C an exothermic effect of about 125 J/gram is found, indicating decomposition or evaporation. Heating of the compound above the phase transition temperature invariably results in non-reproducibility of the thermogram.

#### 4.9 Solubility

Mitoxantrone hydrochloride is sparingly soluble in water, slightly soluble in methanol, practically insoluble in acetonitrile and insoluble in chloroform and acetone (37). Solubility definitions are according to the US Pharmacopeia XX page 1121.

#### 4.10 Partition Coefficient

Extraction properties of mitoxantrone have been investigated for isolation purposes from biological fluids (45).

Direct extraction from aqueous buffer solutions (pH 7.4) with several extractants was incomplete. Mitoxantrone can be quantitatively extracted from pH 4.0 buffer into 1-pentanol as a heptafluorobutyrate ion aggregate (45).

#### 4.11 Dissociation Constants

The mitoxantrone molecule contains several prototropic functions. The 1,4-hydroquinone moiety possesses acidic properties. The two nitrogen atoms attached to the tricyclic aromatic skeleton and the secondary nitrogens in the side chains can accept protons. No full characterization of the prototropic functions in mitoxantrone has been reported, so far. Two  $pK^a$  values, of 5.99 and 8.13, have been mentioned in the literature but they were not assigned to specific functions in the mitoxantrone molecule (46).

#### 4.12 Electrochemistry

The molecular structure of mitoxantrone contains several electrochemically active functions. Although the redox properties of the individual groups, as parts of other compounds e.g. doxorubicin, have been thoroughly investigated (47-54), no in-depth analyses on the electrochemistry of mitoxantrone have been published, hitherto.

Haupt and Baldwin (55) reported on the electrochemical behavior of mitoxantrone by cyclic voltammetry.

Direct current polarographic curves for mitoxantrone hydrochloride are shown in Figure 15 (56). The polarographic curves were recorded on a Bruker E 310 modular electrochemical system, equipped with a drop-timer and Kipp BD 90 x-y recorder. A water-jacketed 10 ml polarographic cell (Metrohm EA 880-T-5) with a dropping mercury electrode, a Metrohm EA 436 Ag/AgCl/3 M KCl reference electrode and a platinum wire auxiliary electrode were employed. The cell was kept at 25°C. The direct current polarograms were recorded at a scan rate of  $2 \text{ mV.s}^{-1}$  and with a drop-time of 2 s. The drug concentration was  $1.0 \times 10^{-4} \text{ M}$ . The compositions of the buffers and solutions used are listed in Table VI.

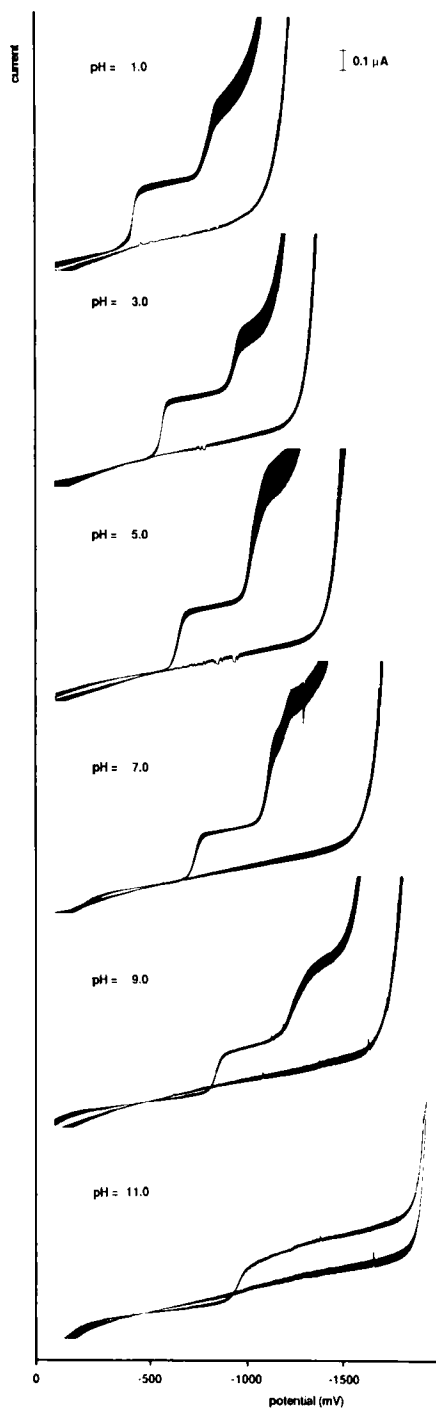


Figure 15

Direct current polarographic curves of mitoxantrone hydrochloride ( $1 \times 10^{-4}$  M)

Table VI Buffers and Solutions used for Direct Current Polarography

pH 1.0	0.1 M sulphuric acid
pH 3.0	0.02 M phosphate buffer ( $\mu$ = 0.1 M, adjusted with potassium chloride)
pH 5.0	0.1 M acetate buffer
pH 7.0	0.1 M phosphate buffer
pH 9.0	0.05 M borate buffer ( $\mu$ = 0.1 M, adjusted with potassium chloride)
pH 11.0	0.1 M phosphate buffer

The registration of the polarographic curves has been performed by generally accepted procedures in electrochemistry (57). The results (Figure 15) indicate a complex pattern of reduction processes. The first wave may correspond with the reduction of the quinone moiety to the corresponding hydroquinone. Although speculative, the second wave in the polarograms, absent in the recording at pH = 11.0, may be a catalytic wave residing the 1,4-dihydroxy-naphthoquinone moiety in the mitoxantrone molecule. The wave disappears at pH = 11.0 which may be due to deprotonation of the phenolic function.

## 5. METHODS OF ANALYSIS

### 5.1 Elemental Analysis

The elemental analysis of mitoxantrone hydrochloride for C, H, N and Cl has been reported (37) as follows:

Table VII Elemental Analysis of Mitoxantrone Hydrochloride (37)<sup>a</sup>

element	% theory	% found
C	48.31	47.96
H	6.07	5.87
N	10.25	10.11
Cl	12.81	12.85

<sup>a</sup> the water content of the sample was determined with a Karl Fischer titration to be 5.39%

### 5.2 Ultraviolet-Visible Spectrophotometry

Analysis of mitoxantrone hydrochloride has been performed with UV-VIS spectrophotometry at 240 and 660 nm in 0.01 M HCl solution. The assays are linear in the concentration range  $4 \times 10^{-6}$  to  $4 \times 10^{-5}$  M, with a precision of  $\pm 1\%$ .

### 5.3 Thin Layer Chromatography

Thin layer chromatographic systems for analysing mitoxantrone have been listed in Table VIII. In most cases mitoxantrone appears as a tailing spot. The mitoxantrone spot can be located by:

- examination under daylight as mitoxantrone has a dark-blue colour;
- irradiation with UV light of 254 nm.

Table VIII      Thin Layer Chromatography of Mitoxantrone Hydrochloride

sorbent	solvent	Rf
silica gel	methanol-strong ammonia solution (100:1.5, v/v)	0.04
	chloroform-methanol-1 M ammonia (9:3:1, v/v/v) (System II)	0.77
cellulose	formic acid-water (4:96, v/v)	0.33
micro-	methanol-strong ammonia solution	0.53
polyamide	(100:1.5, v/v)	
	formic acid-water (4:96, v/v)	0.92
reversed-phase	dimethylformamide-0.5 M sodium chloride	0.44
KC-18F	solution-1 M ammonia-acetic acid (8:8:1:2, v/v/v/v) (System I)	

Systems I and II (Table VIII) have been designed for purity control (37). Solvent system I was used to separate known related compounds from mitoxantrone and detect any unknowns with high Rf values. Solvent system II was used to separate several related compounds from each other and detect any unknowns with low Rf (37).

### 5.4 High Performance Liquid Chromatography

HPLC has turned out to be the most appropriate analytical tool for analyzing mitoxantrone in biological matrices (39,45,55,58-62) and for purity determinations (37). Detection by UV-VIS absorbance at 242 nm (37), 254 nm (45,63), 546 nm (39), 611 nm (59) and 658 nm (58,60,61) is employed.



In general, detection at 658 nm is preferred as this detection mode offers the advantages of high sensitivity and specificity. On-line electrochemical detection (ECD) by oxidation at a glassy carbon electrode at 800 mV is an alternative for mitoxantrone detection following HPLC (39). The advantage of this amperometric detection mode is that a totally different type of selectivity is obtained compared with UV-VIS detection, which may be favourable in e.g. the search for metabolites and degradation products. However, ECD yields no real advantages in terms of sensitivity.

A systematic study on the chromatographic behavior of mitoxantrone and related bis(substituted aminoalkylamino)anthraquinones in HPLC has been reported by Taylor and Gaudio (64). Normal phase systems appeared to be inappropriate, but gradient-eluting reversed phase chromatography with  $\mu$ -Bondapak CN and  $\mu$ -Bondapak NH<sub>2</sub> columns proved to be suitable for aminoanthraquinone analysis. Resolution and peak shapes greatly enhanced and retention times decreased when reversed-phase columns at increased temperatures (up to 50°C) were utilized (64). Ion-pair chromatography is also very suitable for mitoxantrone analysis (37,45,59-61). An improvement in resolution on increasing the column temperature from 25 up to 50°C, also occurred in these systems. This was evidenced by the emergence of four extra peaks (impurities and chemical degradation products) which co-eluted with mitoxantrone at 25°C (45).

Reynolds, Sternson and Repta (45) selected a system comprising a mobile phase of methanol-0.05 M ammonium dihydrogen phosphate buffer (pH 2.7) (45:55) made 6 mM in sodium octane sulfonate and a  $\mu$ -Bondapak C18 column for obtaining maximum resolution of mitoxantrone, chemical degradation products and impurities within a minimum analysis time. Important HPLC systems for mitoxantrone analysis are enumerated in Table IX.

Table IX High Performance Liquid Chromatography for Mitoxantrone Hydrochloride

Column	Mobile phase/column temperature	reference
$\mu$ -Bondapak CN (10 $\mu$ m)	gradient of 20-90% methanol in 0.05 M phosphate buffer (pH 8.0) within a 15 min. period; column temperature 50°C	(64)
$\mu$ -Bondapak NH <sub>2</sub> (10 $\mu$ m)	gradient of 10-90% acetonitrile-methanol (1:1) in 0.05 M ammonium acetate-ammonia buffer (pH 8.0); column temperature 50°C	(64)
$\mu$ -Bondapak C <sub>18</sub>	4.4 M ammonium formate buffer (pH 4.3)-acetonitrile-water (2:1:1); ambient temperature	(39)
$\mu$ -Bondapak C <sub>18</sub> (10 $\mu$ m)	methanol-0.05 M ammonium dihydrogen phosphate buffer (pH 2.7)(45:55) containing 6 mM sodium octanesulfonate; column temperature 49 $\pm$ 1°C	(45)
$\mu$ -Bondapak C <sub>18</sub> (10 $\mu$ m)	acetonitrile-0.2 M ammonium acetate buffer (pH 4.0)(25:75); ambient temperature	(58)
$\mu$ -Bondapak C <sub>18</sub> (10 $\mu$ m)	methanol-0.05 M phosphate buffer (pH 7)(45:55, v/v) containing 0.06% of octilinium bromide and adjusted to pH 4.35 by addition of 6 M hydrochloric acid	(59)
$\mu$ -Bondapak C <sub>18</sub> (10 $\mu$ m)	acetonitrile-0.16 M ammonium formate buffer (pH 2.7)(30:70) containing 25 mM hexane-sulphonic acid	(60)
$\mu$ -Bondapak C <sub>18</sub> (10 $\mu$ m)	acetonitrile-water (25:75) containing 5 mM 1-pentane-sulphonic acid; ambient temperature	(61)
$\mu$ -Bondapak phenyl (10 $\mu$ m)	acetonitrile-water (25:75, v/v) plus 25 ml of PIC B-7 reagent (Waters Assoc.) per liter; ambient temperature	(37)

### 5.5 Identification and Purity Tests in Official Compendia

Inherent to its recent introduction mitoxantrone hydrochloride has not been inserted yet into International or National Pharmacopeias. The manufacturer (Lederle Laboratories, Pearl River, New York, USA) handles the requirements and specifications for mitoxantrone hydrochloride raw material as given in Table X (37).

Table X                      Tests and Specifications for Mitoxantrone Hydrochloride

test	specification
description	dark-blue powder
water	not more than 10%
heavy metals (as Pb)	not more than 20 parts per million
identification (infrared)	the infrared spectrum of sample corresponds to that of a known standard
ethylalcohol (GLC)	not more than 1.5% (w/w)
pyrogens	meets requirement of test
microbial limit	not more than 100 micro-organisms per gram
assay (HPLC)	not less than 82.5% or more than 87.6% mitoxantrone calculated on the anhydrous basis
related compounds (HPLC)	not more than 1.5% of any one related compound by area percent. Not more than 3.0 % total related compounds by area percent

## 6. STABILITY, DEGRADATION

### 6.1 Stability in Aqueous Solution

Mitoxantrone hydrochloride, in its solid state, is a very stable compound. The bulk powder stored at 42°C for four months, at 37°C for six months and at 23°C for 57 months did not change in potency, measured by using a stability-indicating HPLC procedure (37). Furthermore, the content and physical appearance of mitoxantrone hydrochloride powder, stored in flint glass vials, were not affected by natural sunlight exposure for one month. The stability of mitoxantrone hydro-

chloride, when stored in aqueous solution, is limited. Reynolds et al. (45) found mitoxantrone hydrochloride to be unstable in 0.05 M phosphate buffer (pH 7.4) at room temperature. They calculated a half-life of about 130 hours from a (pseudo) first order plot. Preliminary results from another study, performed at 50°C also showed that the degradation of the drug in buffers can be adequately described by (pseudo) first order kinetics. The degradation rate increases at higher pH values indicating the catalytic potency of hydroxyl ions (65). Systematic studies on the degradation kinetics of mitoxantrone hydrochloride have not been finished yet. Structures of degradation products are shown in Figure 16 (37).

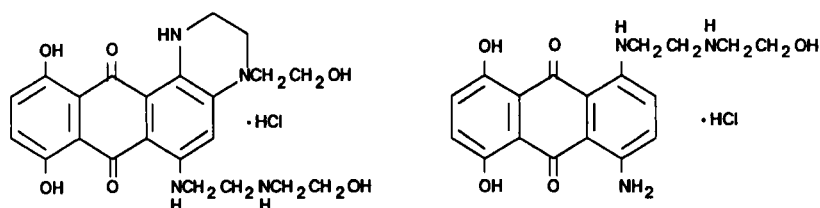


Figure 16  
Structures of degradation products of mitoxantrone

## 6.2 Stability in Pharmaceutical Formulations

Mitoxantrone hydrochloride is supplied as a sterile dark blue solution in vials containing per 10 ml: 20.0 mg mitoxantrone (as hydrochloride salt), 80 mg sodium chloride, 0.5 mg sodium acetate, 4.6 mg glacial acetic acid and Water for Injection ad 10.0 ml. The pH of this parenteral solution lies between 2.0 and 4.5 (46). Vials of 10 and 12.5 ml injection fluid are available under the trade name Novantrone®. The manufacturer indicates that the intact vials are stable for at least three years when stored at room temperature (37). After exposure to natural sunlight for one month, the solutions showed no deterioration (37). For i.v. administration, the drug solution is admixed with 0.9% sodium chloride or 5% dextrose solution. These mitoxantrone parenteral solutions, at 0.01 to 0.5 mg/ml concentrations, are physically and che-

mically stable for at least 48 hours at room temperature (37,66).

### 6.3 Stability in Biological Media

Work by Reynolds et al. (45) and Peng et al. (58) has shown that mitoxantrone is not stable in biological media. Knowledge about this instability is of paramount importance as drug loss from plasma samples can lead the researcher to erroneous results and misinterpretations. The results from the abovementioned studies have been tabulated (Table XI).

Table XI

Stability of Mitoxantrone Hydrochloride in Plasma

conditions	stability	reference
plasma, pH 7.4, 25°C	$t_{1/2} \sim 24$ hours	(45)
plasma, pH 7.4, 4°C	$t_{1/2} \sim 6$ days (60% loss in one week)	(45)
plasma, pH 7.4, -17°C	$t_{1/2} \sim 36$ days (13% loss in one week)	(45)
plasma, pH 5.3, 25°C	$t_{1/2} \sim 60$ hours	(45)
plasma + 2.6% (w/v) sodium bisulfite, pH 7.4, 25°C	$t_{1/2} \sim 20$ minutes	(45)
plasma + 0.5% (w/v) ascorbate, pH 5.3, 25°C	less than 1% loss in 48 hours	(45)
plasma + 0.5% (w/v) ascorbate, pH 5.3, 4°C	less than 4% loss in one week	(45)
plasma, 25°C	60% loss in 24 hours	(58)
plasma, 37°C	80% loss in 24 hours	(58)
plasma proteins (5% human albumin + 3% $\gamma$ -globulin), 25°C	20% loss in 24 hours	(58)
plasma proteins (5% human albumin + 3% $\gamma$ -globulin), 37°C	40% loss in 24 hours	(58)
plasma + 1% (v/v) of 5% L-ascorbic acid in citrate buffer (0.1 M, pH 3.0), 37°C	less than 10% loss in 24 hours	(58)

Oxidation, chemical degradation and irreversible interaction of mitoxantrone with plasma protein may account for the drug instability (45,58). Nevertheless, it should be clear that stabilization of plasma samples is imperative. Freshly obtained plasma samples should be immediately fortified with ascorbic acid and the pH must be adjusted to 5.3. Furthermore, the samples must be kept frozen prior to analysis.

## 7. PHARMACOLOGY

### 7.1 Mechanism of Action

The exact mechanism by which mitoxantrone exerts cytotoxicity has not been elucidated yet. Like doxorubicin and related anthracyclines, the drug intercalates into the DNA helix leading to an inhibition of nucleic acid synthesis (67, 68). DNA binding is independent of the stage of cell cycle (69,70). Although the structure of the mitoxantrone-DNA complex is not fully understood, it can be expected that, in analogy with doxorubicin, DNA binding is facilitated by the planar, tricyclic, electron-rich chromophore. The binding may be strengthened by the presence of the aliphatic amino group, which can be expected to be protonated under physiological conditions and which can bind electrostatically with the phosphate groups of DNA (67).

As there seems to exist no correlation between mitoxantrone-DNA binding and antitumour activity other mechanisms than intercalation are obvious (19,67). However, these mechanisms of action remain to be elucidated.

### 7.2 Clinical Antitumour Activity

Most clinical experience with mitoxantrone exists in the treatment of patients suffering from advanced breast cancer. The response rate is from 30-36% in these patients with minimal prior therapy (33). The recommended dosage regimen, concluded from phase I studies, for the treatment of breast carcinoma is 12-14 mg/m<sup>2</sup> given i.v. once every 21 days. In studies where mitoxantrone and doxorubicin have been compared in breast cancer, the drugs show a comparable clinical efficacy in terms of duration of response, total response rate and survival (71-74). Single agent activity of mitoxantrone has been evaluated in a great number of phase II trials. Responses have been noticed not only in breast cancer but also in the following tumor types: endometrium (76), head and neck (75), liver (77,78), lymphoma (79) and acute non-lymphocytic leukemia (80,81). Clinical usefulness of mitoxantrone has also been studied when the drug has been incorporated into combination regimens (83-90).

In a study where CAF (cyclophosphamide, doxorubicin, 5-

fluoro-uracil) and CNF (cyclophosphamide, mitoxantrone, 5-fluoro-uracil) were compared both regimens turned out to be equal in terms of responses (91). Intraperitoneal administration of mitoxantrone for the treatment of minimal residual disease in ovary cancer is currently investigated clinically (92,93).

### 7.3 Clinical Toxicity

Intravenous administration of mitoxantrone is generally well tolerated and relatively free from serious side-effects. Phase I trials have shown that myelosuppression, in particular leukopenia, is the dose limiting toxicity (20,94,95). The most predominant non-haematological toxicities include: nausea, vomiting, stomatitis and alopecia but these are rarely severe (96). In comparison with doxorubicin, at equi-active dosages, the toxicities of mitoxantrone appear to be milder. The potential for cardiac toxicity of mitoxantrone receives a great deal of attention (33). This is because the compound was initially developed as an anthracycline analog with reduced cardiotoxicity. So far, it can be concluded that mitoxantrone is certainly not a non-cardiotoxic drug but the risk for cardiotoxicity is lower than for doxorubicin. Pre-existing factors for evoking cardiac complications after mitoxantrone therapy are: cardiac diseases, prior anthracycline chemotherapy and mediastinal radiotherapy (97,98). Accidental mitoxantrone extravasation shows in most cases no complication (20,95) in terms of tissue necrosis, although one report has been published (99) in which extravasation led to damage of the soft tissues around the injection site.

### 7.4 Pharmacokinetics

Plasma pharmacokinetic studies of mitoxantrone have shown that the drug is taken up rapidly by tissues, followed by a very slow redistribution back into the plasma and elimination from the body (34,100-106). The plasma concentration-time curves of i.v. treated patients fits best to an open three compartment model. Pharmacokinetic parameters from the study of Van Belle *et al.*, (103) have been listed in Table XII. This study concerned 21 patients who were treated i.v. with mitoxantrone (15 mg/m<sup>2</sup> over 30 minutes).



Tabel XII      Pharmacokinetic Data of I.V. Treated Patients (103)

parameter	mean value
peak	683 ng/ml
$t_{1/2\alpha}$	0.206 h
$t_{1/2\beta}$	1.55 h
$t_{1/2\gamma}$	36.14 h
AUC/mg	36.64 ng ml <sup>-1</sup> mg <sup>-1</sup>
V-central	26.22 L
V-distribution	1381.9 L
Urinary excretion	4.92% of total dose/24 h

Stewart et al. (62) have measured mitoxantrone concentrations in autopsy tissue samples of patients who had received the drug i.v. 10-272 days antemortem. The highest concentrations appear to attain in thyroid, liver and heart. Concentrations in tumour tissues were generally lower than in normal tissues.

Hepatobiliary excretion constitutes the primary route of drug elimination. Only a low amount is excreted in the urine and a variable quantity is excreted in bile. In both animals and man mitoxantrone is metabolized to the mono- and dicarboxylic acid derivatives (Figure 17) as well as the glucuronide conjugates of these acids (104,107-109). Ehninger et al. (104) also found two additional metabolites in human urine.

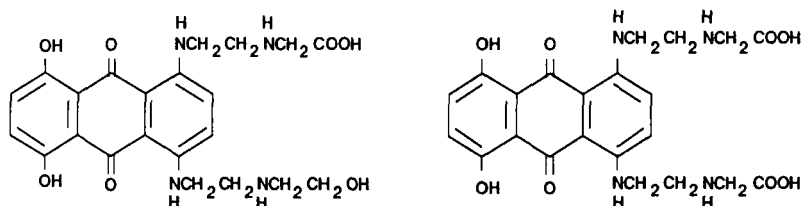


Figure 17

Structures of the mono- and dicarboxylic metabolites of mitoxantrone

## 8. DETERMINATION IN BODY FLUIDS

Mitoxantrone can be determined in serum by a radioimmunoassay (38). The sensitivity of this assay is 50 pg/ml using 0.5 ml samples. The advantages of this method are the high sensitivity and the fact that no sample pretreatment is required. However, the preparation of  $^3\text{H}$ -mitoxantrone radioactive tracer and the antiserum makes the assay not useful for routine analysis in every laboratory. The analysis of mitoxantrone in biological samples is usually performed with HPLC. The structure analog ametantrone has been used with success as internal (59,60) standard. The structure of this compound is shown in Figure 18.

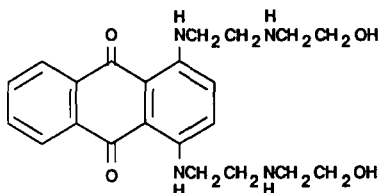


Figure 18  
Structure of ametantrone

Reported sample pretreatment procedures are liquid-liquid extraction (39,59,60,62) and liquid-solid extraction (45,55,58,61). Liquid-liquid extraction is usually performed with an alkyl-sulphonic acid in order to extract mitoxantrone as ion-pair with the counter ion into the organic solvent. Protein precipitation methods with trichloro-acetic acid or perchloric acid lead to very poor recoveries (39). Some authors prefer the use of silanized glass-ware to prevent drug binding to glass surfaces (39,60).

The HPLC assay designed by Van Belle *et al.* (103), has proven to be suitable for pharmacokinetic studies. The following procedure is applied for plasma samples: 10 ml sample was added to 1 ml solution containing hexane-sulphonic acid (0.01 mg/ml), ascorbic acid (0.5 mg/ml) and ametantrone (1.2 mg/ml). After vortex mixing, 1 ml 0.1 M borax buffer (pH 9.5) was added with subsequent extraction with 5 ml dichloromethane. After centrifugating at 1500 g, the organic layer was separated and the solvent evaporated under nitrogen. The dry

residue was reconstituted with the mobile phase and injected into the chromatograph. Urine samples could be injected directly into the chromatograph after addition of the internal standard. HPLC: column:  $\mu$ -Bondapak C<sub>18</sub> (30 cm long x 3.9 mm i.d., particle size 10  $\mu$ m); mobile phase: acetonitrile - 0.16 M ammonium formate buffer pH 2.7 (30:70) containing 25 mM hexane-sulphonic acid; flow rate: 1.0 ml/min; detection: 658 nm; temperature: ambient.

The method of Ehniger et al. (61,104) allows the measurement of mitoxantrone and four metabolites in serum and urine. The sample pretreatment involves a liquid-solid extraction. The procedure is: disposable columns from Pasteur capillary pipettes were prepared with 150 mg of XAD-2 beads suspended in 2 ml of methanol. The pipettes were closed in the tip and at the top with a quartz wool plug. The columns were washed with water and 0.05 M phosphate buffer pH 7.4. Volumes of 1-10 ml stabilized serum (through the immediate addition of 5% (w/v) of ascorbic acid in 0.1 M citrate buffer (pH 3)(9 parts of serum + 1 part of stabilizer)) were applied to the XAD-2 columns and washed with two 2 ml portions of 0.05 M ammonium dihydrogen phosphate solution (pH 2.7). Then the column was washed with 0.2 ml of 2-propanol-0.05 M ammonium dihydrogen phosphate buffer (pH 2.7)(30:70 v/v) after which mitoxantrone and its metabolites were eluted with a further 0.4 ml of this solution. A 0.1 ml volume was injected into the chromatograph. Volumes of 10-30 ml of urine were applied directly to the XAD-2 columns, washed with two 2 ml portions of 0.05 M ammonium dihydrogen phosphate solution (pH 2.7) and extracted with 1 ml of 2-propanol-0.05 M ammonium dihydrogen phosphate buffer (pH 2.7) (30:70, v/v) into glass microvials and volumes of 0.1 ml were injected into the HPLC system. HPLC: column:  $\mu$ -Bondapak C<sub>18</sub> (30 cm long x 3.9 mm i.d., particle size 10  $\mu$ m) + guard column filled with Co:Pell ODS material; mobile phase: acetonitrile-water (25:75) containing 5 mM 1-pentane-sulphonic acid; flow rate: 1.0 ml/min; detection: 658 nm; temperature: ambient.

An overview of published determination methods of mitoxantrone in biological materials is given in Table XIII.

Table XIII

## HPLC Determination Methods for Mitoxantrone in Biological Matrices

matrix	sample pretreatment <sup>a</sup>	internal <sup>b</sup> standard	determination limit	reference
plasma, serum, urine	p.p. + l.l.	yes	-	(39)
plasma	l.s.	no	75 ng/ml	(45)
plasma	l.s.	no	1 ng/ml	(58)
plasma	l.l.	yes	3 ng/ml	(59)
plasma	l.l.	yes	1 ng/ml	(60)
urine	no	yes	-	(60)
serum	l.s.	no	1 ng/ml	(61)
urine	l.s.	no	0.2 ng/ml	(61)
urine	l.s.	no	0.4 ng/ml	(55)
tissue	l.l.	yes	2 ng/g	(62)

<sup>a</sup> p.p.: protein precipitation; l.l.: liquid-liquid extraction; l.s.: liquid-solid extraction

<sup>b</sup> the internal standard is ametantrone (59,60,62) or cresyl violet (39)

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# ANALYTICAL PROFILE OF MORPHINE

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# MORPHINE

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## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

- Morphinan-3,6-diol,7,8-didehydro-4,5-epoxy-, 17-methyl-(5 $\alpha$ ,6 $\alpha$ ).
- 7,8-Didehydro-4,5-epoxy-17-methyl morphinan-3,6-diol.
- 7,8-Didehydro-4,5 $\alpha$ -epoxy-3,6 $\alpha$ -dihydroxy-N-methyl-morphinan.
- (5R,6S)-4,5-epoxy-N-methylmorphin -7-ene-3,6-diol.
- (4aR,5S,7aR,8R,9cS)-4a,5,7a,8,9,9c-Hexahydro-12-methyl-8,9c-iminoethanophenanthro[4,5-bcd]furan-3,5-diol.
- Morphinan-3,6-diol,7,8-didehydro-4,5-epoxy-17-methyl-(5 $\alpha$ ,6 $\alpha$ )-, hydrochloride (Morphine hydrochloride).
- Morphinan-3,6-diol, 7,8-didehydro-4,5-epoxy-17-methyl-(5 $\alpha$ ,6 $\alpha$ )-, sulfate (2:1) (salt), pentahydrate (Morphine sulfate).

#### 1.1.2 Generic Names

Morphine, Morphina, Morphia, Morphium.

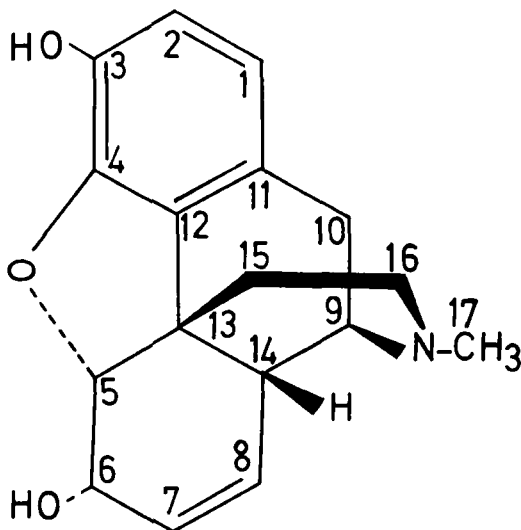
#### 1.1.3 Trade Names for morphine sulfate

MS contin; Duramorph PF (preservative free injection); Roxanol (concentrated oral solution).

### 1.2 Formula

#### 1.2.1 Empirical

$C_{17}H_{19}NO_3$	(Morphine)
$C_{17}H_{20}Cl NO_3$	(Morphine hydrochloride)
$C_{34}H_{50}N_2O_{15}S$	(Morphine sulfate pentahydrate)
$C_{34}H_{40}N_2O_{10}S$	(Morphine sulfate anhydrous)

1.2.2 Structural

More than twenty structures were proposed for morphine and the currently accepted structure is that proposed in 1925 by Gulland and Robinson (1). The proposed structure was confirmed by the total synthesis of morphine which was achieved in 1956 by Gates and Tschudi (2).

1.2.3 CAS Registry Number

Morphine [57-27-2]

Morphine hydrochloride [52-26-6]

Morphine sulfate (anhydrous) [64-31-3]

Morphine sulfate (pentahydrate) [6211-15-0]

1.2.4 Wiswesser Line Notations (3)

T B6 566 B6/CO \_4ABBC R BX H

^O PN DU GHT && TTJ FQ J QP

T B6 566 B6/CO 4ABBC R BX H

^O PN DU GHT && TTJ FQ JQ P

& GH & QH 3 (Morphine hydrochloride trihydrate)

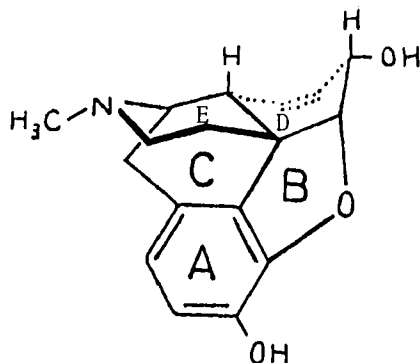
### 1.2.5 Stereochemistry

Morphine possesses five chiral centres (at C<sub>5</sub>, C<sub>6</sub>, C<sub>9</sub>, C<sub>13</sub> and C<sub>14</sub>), but since the bridged ring system must be *cis*, eight pairs of enantiomers are possible (4). The deduction of relative configurations at the various centres by chemical methods has been well summarized by Ginsburg (5).

The absolute stereochemistry has been deduced from a combination of X-ray crystallography and chemical degradation and correlation. From the X-ray data of morphine hydrate (6), morphine hydrochloride trihydrate (7), morphine iodide dihydrate (8) and codeine hydrobromide dihydrate (9,10) it was concluded that the molecules of morphine and codeine are approximately T-shaped, and each molecule consists of two roughly planar parts. The first plane involves the atoms C<sub>1</sub> through C<sub>5</sub>, C<sub>9</sub> through C<sub>14</sub>, C<sub>3</sub>-OH and C<sub>4</sub>-O comprising the benzene ring [A], the oxide ring [B] and the carbocyclic ring [C]. The second plane consists of the carbocyclic ring [D] and the ethanamine ring [E] and includes the atoms C<sub>5</sub> through C<sub>9</sub>, C<sub>13</sub> through C<sub>17</sub>, C<sub>6</sub>-OH and N. The two planes are nearly perpendicular (90.9°) (7).

The atoms at the benzene ring [A] are nearly coplanar, whereas the five membered ring [B] is distorted, ring [C] is a halfchair conformer (6). The ethanamine ring [E] has a typical chair form, while ring [D] has a boat form. The C<sub>5</sub> - oxygen and C<sub>6</sub> - hydroxyl are *cis* to each other.

The absolute configuration of (-)-morphine can therefore be written as follows:-





### 1.3 Molecular Weight

285.33	(morphine)
321.81	(morphine hydrochloride)
668.76	(morphine sulfate)

### 1.4 Elemental Composition

C, 71.56%; H, 6.71%; N, 4.91%; O, 16.82%. (morphine).  
C, 63.45%; H, 6.26%; Cl, 11.02%; N, 4.35%; O, 14.92%.  
(morphine hydrochloride).  
C, 61.06%; H, 6.03%; N, 4.19%; O, 23.93%; S, 4.79%.  
(morphine sulfate).

### 1.5 Appearance, Color, Odor and Taste

Short shining orthorhombic colorless prisms, or fine colorless needles (from methanol) or white crystalline powder, odorless. It has a bitter taste (morphine).

Colorless silky crystals or cubical white masses or white flakes or white crystalline powder, odorless and has a bitter taste.

The trihydrate salt loses its water of crystallisation at about 100° and usually becomes yellowish (morphine hydrochloride).

White acicular crystals or cubical masses or white feathery silky crystals or a white crystalline powder, odorless and has a bitter taste. Darkens on prolonged exposure to light (morphine sulfate).

### 1.6 Loss on Drying

When morphine hydrochloride is dried to constant weight at 130° it losses 12% to 15% of its weight (use 0.5g) (11).

When morphine sulfate is dried at 145° for one hour, it loses not less than 9.0% and not more than 12% of its weight (11).

## 2. Physical Properties

### 2.1 Melting Range

Morphine melts at 254-256.4° (with decomposition).

Morphine hydrochloride melts at about 200° (with decomposition).

Morphine sulfates melts at about 250° (with decomposition).

A metastable phase mp 197° (morphine).

Morphine sublimates at 190-200° (0.2 mm pressure at 2 mm distance (12)).

### 2.2 Eutectic Temperature (13)

	Morphine hydrochloride	Morphine sulfate
Sal.	189°	185°
Dic.	179°	150°
Phenolph.	235°	217°

---

Sal = acetaminosalol, Dic = dicyandiamide,  
Phenolph = phenolphthalein.

### 2.3 Solubility

- One gram of morphine dissolves in about 5000 ml water, 1100 boiling water, 210 ml alcohol, 98 ml boiling alcohol, 1220 ml chloroform, 6250 ml ether, 114 ml amylalcohol, 10 ml boiling methanol, 525 ml ethyl-acetate.

Freely soluble in solutions of alkaline earth hydroxides, in phenol, cresols; moderately soluble in mixtures of chloroform with alcohols; slightly soluble in benzene and ammonia (12).

- One gram of morphine hydrochloride dissolves in 17.5 ml water, 0.5 ml boiling water, 52 ml alcohol, 6 ml alcohol at 60°, slowly soluble in glycerol, insoluble in chloroform and in ether (12).

BP 1980 (11) reported the following solubility data for morphine hydrochloride:-

One part is soluble in 24 parts of water and in 10 parts of boiling ethanol (90%), soluble at 15° in 100 parts of ethanol (96%) and at 10° in 50 parts of ethanol (90%).

- One gram of morphine sulfate dissolves in 15.5 ml water at 25°, 0.7 ml water at 80°, 565 ml alcohol,

240 ml alcohol at 60°, insoluble in chloroform and ether (12).

The BP 1980 (11) reported the following solubility data for morphine sulfate:

one part is soluble in 21 parts of water and in 1000 parts of ethanol (96%).

#### 2.4 pH Range

Morphine 8.5 (saturated solution)

Morphine hydrochloride about 5.0

Morphine sulfate approximately 4.8

#### 2.5 Dissociation Constant

pKa (morphine) = 9.85 (12)

pKa (morphine) = 6.50 (14)

pKa (morphine hydrochloride) = 8.0, 9.9 at 20° (15).

#### 2.6 Specific Optical Rotation

Morphine

$[\alpha]_D^{25}$  -132° (1% in methanol) (12)

$[\alpha]_D^{23}$  -130.9° (in methanol) (16)

$[\alpha]_D$  -116° (in dilute H<sub>2</sub>SO<sub>4</sub>) (16)

Morphine hydrochloride

$[\alpha]_D^{25}$  -113.5° (c = 2.2 in H<sub>2</sub>O) (12)

$[\alpha]_D$  about -112° (2% w/v solution) (11)

$[\alpha]_D^{18}$  -97.9° (water) (14)

Morphine sulfate

$[\alpha]_D^{25}$  -108.7 (c = 4 in H<sub>2</sub>O anhydrous basis) (12).

$[\alpha]_D$  between - 107° and - 109.5° (200 mg in 10 ml solution, anhydrous basis) (17).

#### 2.7 Crystal Structure

The crystal structure of morphine was determined by X-ray diffraction technique which was achieved by several authors (6-10). The crystals of (-) morphine hydrate, C<sub>17</sub>H<sub>19</sub>NO<sub>3</sub> · H<sub>2</sub>O are orthorhombic with space group  $P2_12_12_1$  and with cell dimensions  $a = 7.438$ ,

$b=13.751$ ,  $c=14.901$  Å. The water molecule bridges the phenolic and hydroxylic oxygen atoms through two O-H...O bonds (6).

The crystals of (-) morphine hydrochloride trihydrate,  $C_{17}H_{19}NO_3 \cdot HCl \cdot 3H_2O$  are also orthorhombic with space group  $P2_12_12_1$  and with the unit-cell dimensions  $a=13.019$ ,  $b=20.750$ ,  $c=6.941$  Å. The morphine molecules connected by N-H...O hydrogen bonds, form chains about the  $2_1$  axes parallel to  $a$  and the chains are connected via water molecules and chloride ions (7).

The crystals of morphine hydroiodide dihydrate,  $C_{17}H_{19}NO_3 \cdot HI \cdot 2H_2O$  are orthorhombic, with space group  $P2_12_12_1$  and with four molecules per unit cell. Unit-cell dimensions  $a=20.32$ ,  $b=13.02$ ,  $c=6.84$  Å (8).

From the above data as well as from other X-ray parameters, it has been concluded that the morphine molecule consists roughly of two planar parts, the first part includes ring A, B and C and the second part includes ring D and E (the ethanamine ring). The two planes are nearly perpendicular and the morphine molecule has the T-shape (6,7,8). The same shape has been found in codeine molecule (9,10).

From the X-rays analysis of morphine molecule and from the extensive chemical evidence to establish the stereochemical configuration of the morphine molecule (18-25), it was possible to deduce the correct absolute configuration of natural (-) form of morphine as it is presented in Fig.2(8).

The crystal structure of morphine hydrate is presented in Fig.1(6).

Interatomic distances and interbond angles in the morphine molecule are shown in Fig. 3 (7). Intramolecular bond lengths and bond angles of both morphine hydrate and morphine hydrochloride trihydrate are listed in tables 1 and 2 respectively.

## 2.8 X-ray Powder Diffraction

The X-ray diffraction pattern of morphine hydrochloride was determined with a Philips X-ray diffraction spectrogoniometer equipped with PW 1730 generator. X-ray radiation was provided by a copper target (Cu-anode 2000w). High intensity X-ray tube operated at 40KV and 20mA. The monochromator was a single curved crystal monochromator ( $\gamma=1.5918^\circ$ ). The unit was equipped with Philips PM 8210 printing recorder and

Fig. 1. The Crystal structure of Morphine Hydrate

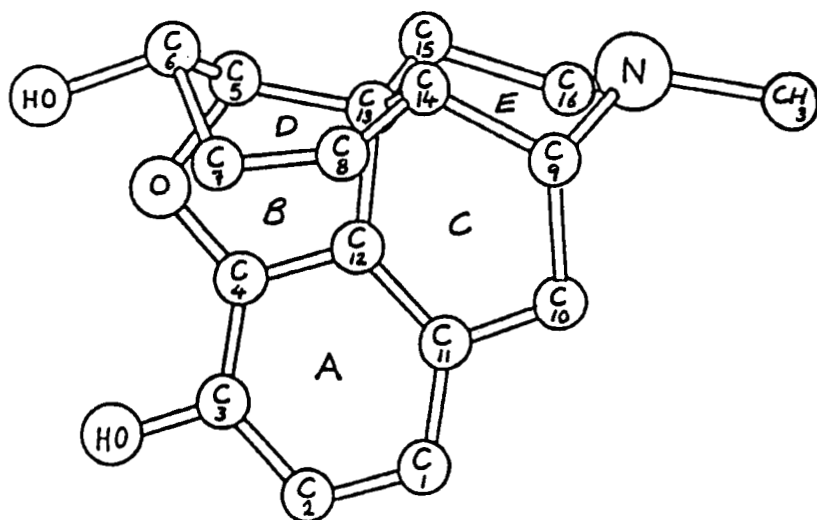
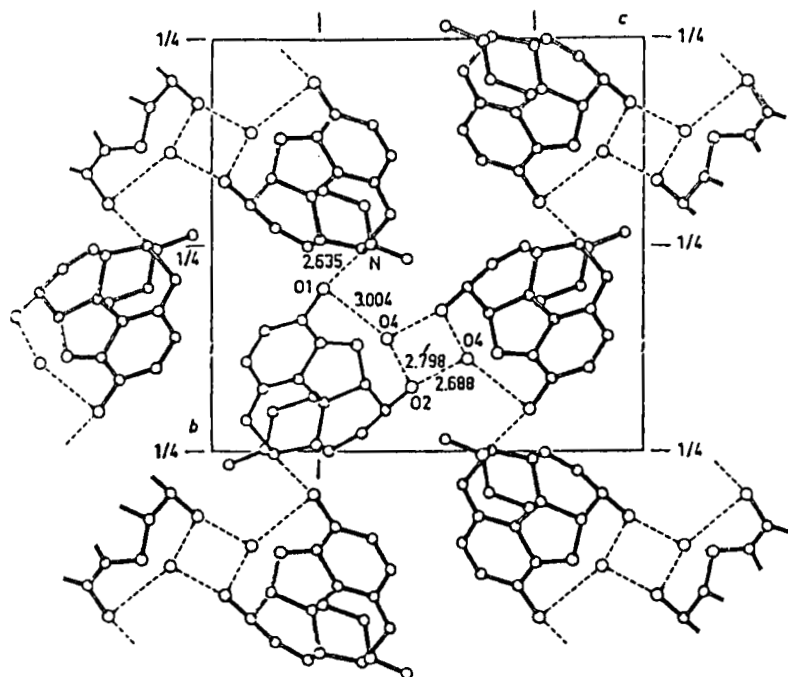


Fig. 2. The Absolute structure of (-)-Morphine



Table 1 : *Intramolecular Bond Lengths (Å) of Morphine Hydrate and Morphine Hydrochloride Trihydrate*

Bond	Morph	Morph HCl	Bond	Morph	Morph HCl
C <sub>1</sub> - C <sub>2</sub>	1.385	1.41	C <sub>8</sub> - C <sub>14</sub>	1.500	1.51
C <sub>1</sub> - C <sub>11</sub>	1.396	1.39	C <sub>9</sub> - C <sub>10</sub>	1.556	1.54
C <sub>2</sub> - C <sub>3</sub>	1.422	1.39	C <sub>9</sub> - C <sub>14</sub>	1.535	1.55
C <sub>3</sub> - O <sub>1</sub>	1.359	1.37	C <sub>9</sub> - N	1.476	1.53
C <sub>3</sub> - C <sub>4</sub>	1.387	1.38	C <sub>10</sub> - C <sub>11</sub>	1.519	1.52
C <sub>4</sub> - O <sub>3</sub>	1.361	1.37	C <sub>11</sub> - C <sub>12</sub>	1.373	1.36
C <sub>4</sub> - C <sub>12</sub>	1.367	1.37	C <sub>12</sub> - C <sub>13</sub>	1.509	1.50
C <sub>5</sub> - O <sub>3</sub>	1.470	1.47	C <sub>13</sub> - C <sub>14</sub>	1.541	1.55
C <sub>5</sub> - C <sub>6</sub>	1.526	1.52	C <sub>13</sub> - C <sub>15</sub>	1.535	1.54
C <sub>5</sub> - C <sub>13</sub>	1.553	1.55	C <sub>15</sub> - C <sub>16</sub>	1.526	1.52
C <sub>6</sub> - O <sub>2</sub>	1.413	1.46	C <sub>16</sub> - N	1.475	1.51
C <sub>6</sub> - O <sub>7</sub>	1.509	1.49	N - C <sub>17</sub>	1.476	1.49
C <sub>7</sub> - C <sub>8</sub>	1.313	1.36			

Table 2 : *Intramolecular Bond Angles (°) of Morphine Hydrate and Morphine Hydrochloride Trihydrate*

Bond Angles	Morph	Morph HCl	Bond Angles	Morph	Morph HCl
C <sub>2</sub> - C <sub>1</sub> - C <sub>11</sub>	123.6	121.2	C <sub>1</sub> - C <sub>11</sub> - C <sub>12</sub>	116.9	116.3
C <sub>1</sub> - C <sub>2</sub> - C <sub>3</sub>	122.2	120.6	C <sub>10</sub> - C <sub>11</sub> - C <sub>12</sub>	117.9	118.7
C <sub>2</sub> - C <sub>3</sub> - C <sub>4</sub>	115.5	117.2	C <sub>4</sub> - C <sub>12</sub> - C <sub>11</sub>	122.4	123.8
C <sub>2</sub> - C <sub>3</sub> - O <sub>1</sub>	123.9	124.4	C <sub>4</sub> - C <sub>12</sub> - C <sub>13</sub>	109.5	108.2
O <sub>1</sub> - C <sub>3</sub> - C <sub>4</sub>	123.3	118.3	C <sub>11</sub> - C <sub>12</sub> - C <sub>13</sub>	127.1	127.4
C <sub>3</sub> - C <sub>4</sub> - C <sub>12</sub>	122.2	120.5	C <sub>5</sub> - C <sub>13</sub> - C <sub>12</sub>	100.3	101.6
C <sub>3</sub> - C <sub>4</sub> - O <sub>3</sub>	124.3	125.6	C <sub>5</sub> - C <sub>13</sub> - C <sub>14</sub>	115.7	115.2
O <sub>3</sub> - C <sub>4</sub> - C <sub>12</sub>	113.8	113.8	C <sub>5</sub> - C <sub>13</sub> - C <sub>15</sub>	112.5	111.1
O <sub>3</sub> - C <sub>5</sub> - C <sub>6</sub>	112.4	111.1	C <sub>12</sub> - C <sub>13</sub> - C <sub>14</sub>	106.8	106.7
O <sub>3</sub> - C <sub>5</sub> - C <sub>13</sub>	106.2	104.7	C <sub>12</sub> - C <sub>13</sub> - C <sub>15</sub>	112.5	112.9
C <sub>6</sub> - C <sub>5</sub> - C <sub>13</sub>	113.3	112.9	C <sub>14</sub> - C <sub>13</sub> - C <sub>15</sub>	109.1	109.1
C <sub>5</sub> - C <sub>6</sub> - C <sub>7</sub>	113.2	114.9	C <sub>8</sub> - C <sub>14</sub> - C <sub>9</sub>	113.5	111.5
C <sub>5</sub> - C <sub>6</sub> - O <sub>2</sub>	113.3	111.2	C <sub>8</sub> - C <sub>14</sub> - C <sub>13</sub>	109.6	109.6
O <sub>2</sub> - C <sub>6</sub> - C <sub>7</sub>	129.3	110.0	C <sub>9</sub> - C <sub>14</sub> - C <sub>13</sub>	107.3	106.1
C <sub>6</sub> - C <sub>7</sub> - C <sub>8</sub>	121.2	120.6	C <sub>13</sub> - C <sub>15</sub> - C <sub>16</sub>	111.5	112.1
C <sub>7</sub> - C <sub>8</sub> - C <sub>14</sub>	119.9	118.3	C <sub>15</sub> - C <sub>16</sub> - N	111.3	109.1
C <sub>10</sub> - C <sub>9</sub> - C <sub>14</sub>	112.6	116.1	C <sub>9</sub> - N - C <sub>16</sub>	111.9	112.4
C <sub>10</sub> - C <sub>9</sub> - N	114.9	111.6	C <sub>9</sub> - N - C <sub>17</sub>	112.6	114.4
N - C <sub>9</sub> - C <sub>14</sub>	127.7	105.4	C <sub>16</sub> - N - C <sub>17</sub>	110.3	111.2
C <sub>9</sub> - C <sub>10</sub> - C <sub>11</sub>	114.9	114.0	C <sub>4</sub> - O <sub>3</sub> - C <sub>5</sub>	106.3	106.6
C <sub>1</sub> - C <sub>11</sub> - C <sub>10</sub>	124.4	124.5			



digital printer. Divergence slit and the receiving slit were  $1^\circ$ . The scanning speed of the goniometer used was 0.02  $2\theta$ /sec., recorder full scale was 10,000 counts at recorder speed of 4 mm/ $2\theta$ . The lower level and the upper level of the signal control was 35 and 75 respectively.

The unit was aligned and examined, using a Solicon standard, to attain maximum operation conditions. The X-ray pattern of morphine HCl is presented in Fig. 4. Interplanar distance and relative intensity are tabulated in table 3.

## 2.9 Spectral Properties

### 2.9.1 Ultraviolet Spectra

The UV spectra of morphine monohydrate in ethanol and in 0.1 N sodium hydroxide solution (Fig. 5) were scanned from 200 to 400 nm using Cary 219 Varian spectrometer. Morphine exhibited the following UV data (Table 4).

Table 4 : UV Characteristics of Morphine

<u>Solvent</u>	<u><math>\lambda_{\max}</math>. nm</u>	<u>log<math>\epsilon</math></u>	<u>A(1%, 1cm.)</u>
Ethanol	286	3.2561	59.44
0.1 N NaOH solution ]	250	3.7250	175
	298	3.3605	75.6

Other reported UV spectral data for morphine:

<u>Solvent</u>	<u><math>\lambda_{\max}</math>. nm</u>	<u>A(1%, 1cm)</u>	<u>Ref.</u>
Ethanol	287	55	(26)
0.1 NaOH solution	250.5	255	(26)
	296	116	(26)
0.1 H <sub>2</sub> SO <sub>4</sub>	284	43.4	(26)
Aqueous acid	285	52	(27)
Aqueous alkali	298	92	(27)

### 2.9.2 Infrared Spectrum

The IR spectrum of morphine as KBr-disc was recorded on a Perkin Elmer 580 B Infrared Spectrophotometer to which an infrared data station is attached (Fig. 6).

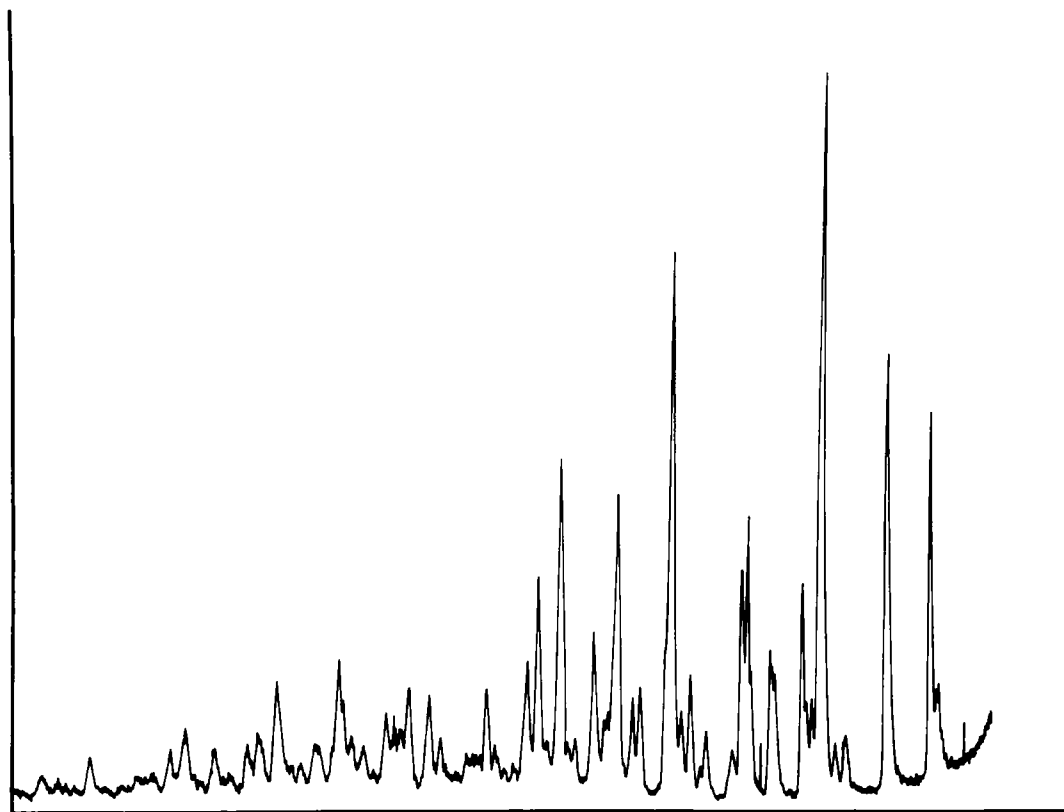


FIG. 4 : THE X-RAY DIFFRACTION PATTERN MORPHINE HYDRATE.

Table 3 : *X-Ray Powder Diffraction Pattern of Morphine Hydrochloride*

d(A°)	I/I <sub>0</sub>	d(A°)	I/I <sub>0</sub>	d(A°)	I/I <sub>0</sub>
12.32	8	3.18	10	2.11	7
11.17	18	3.14	49	2.07	19
10.51	56	3.06	10	2.02	10
8.21	64	3.02	33	2.00	10
6.88	11	2.96	22	1.96	6
6.57	10	2.89	7	1.93	9
6.17	100	2.85	6	1.90	5
6.01	16	2.80	10	1.87	13
5.80	30	2.76	18	1.84	9
5.28	19	2.73	8	1.80	5
4.88	16	2.68	8	1.78	5
4.86	39	2.57	11	1.70	8
4.78	32	2.53	17	1.65	5
4.66	9	2.45	18	1.63	6
4.34	11	2.43	12	1.58	5
4.28	7	2.41	13	1.56	5
4.17	19	2.38	14	1.47	6
4.08	14	2.34	6	1.44	5
3.98	78	2.31	10		
3.70	17	2.27	11		
3.64	15	2.25	16		
3.52	45	2.23	22		
3.43	12	2.22	9		
3.35	25	2.17	10		
3.23	10	2.13	8		

d = interplanar distance, I/I<sub>0</sub> = relative intensity  
(based on the highest intensity of 100).

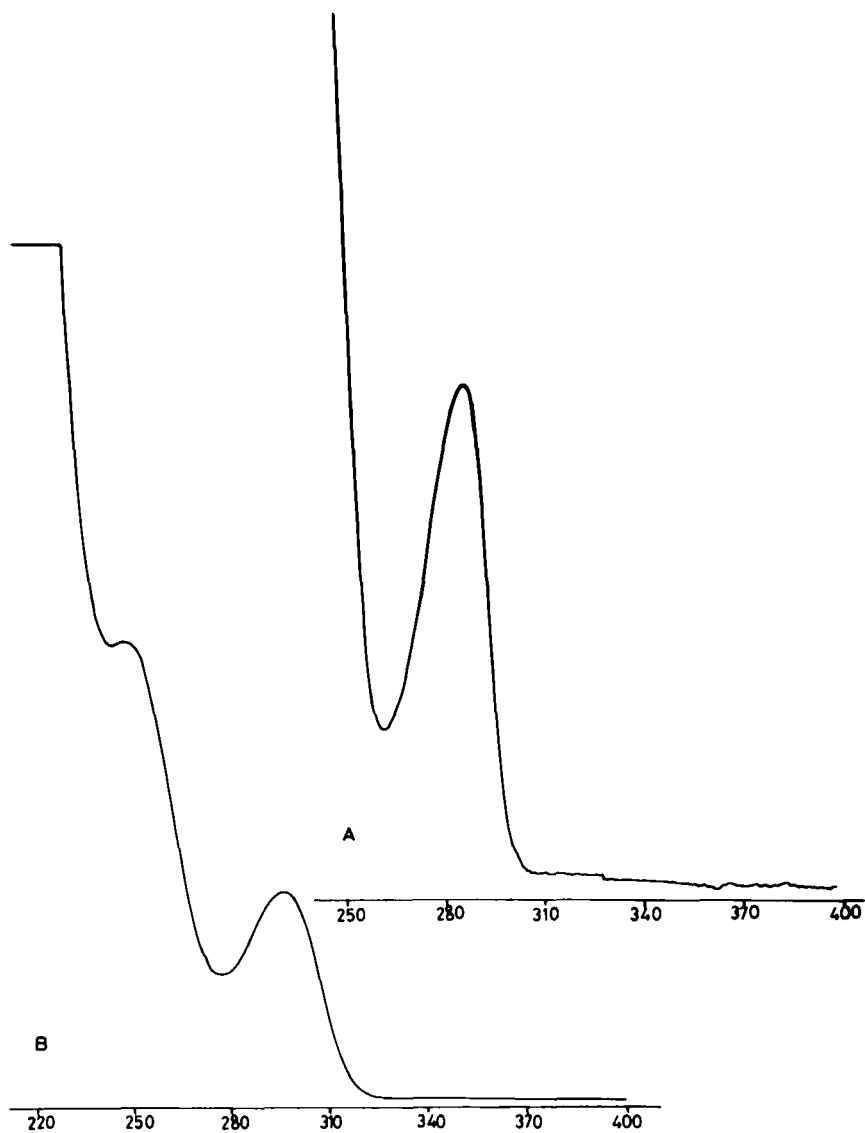


FIG. 5 : THE UV SPECTRA OF MORPHINE A IN METHANOL, B IN 0.1 N NaOH.

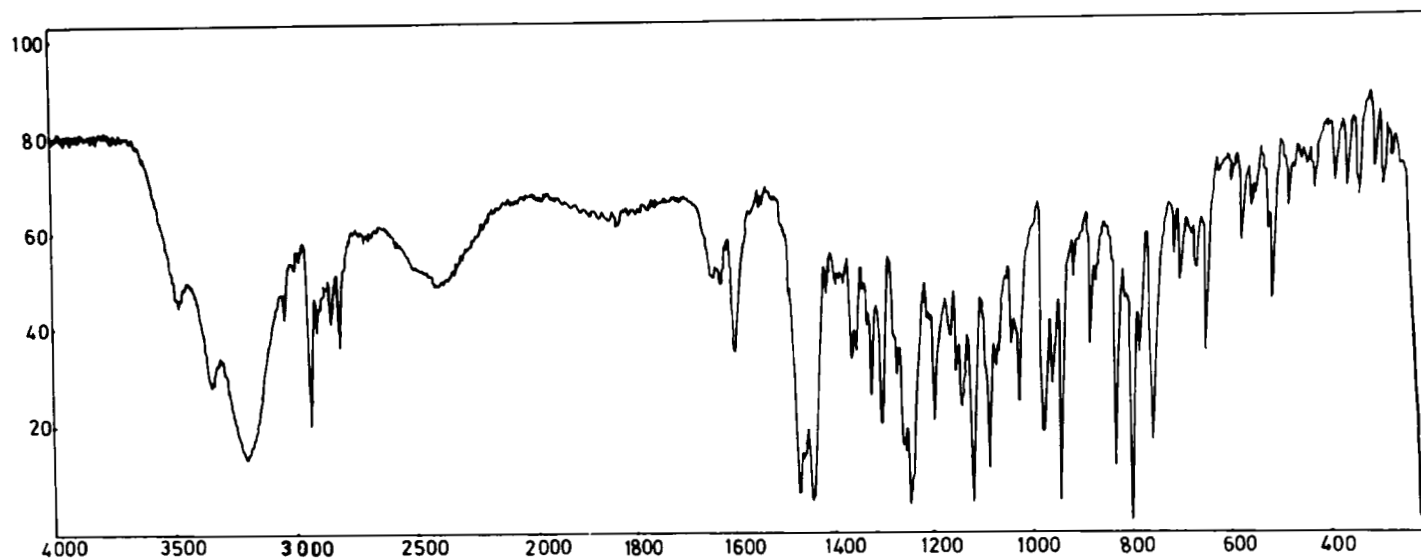


FIG. 6 : THE IR SPECTRUM OF MORPHINE AS KBr DISC.

The structural assignments of morphine have been correlated with the following frequencies (Table 5).

Table 5 : IR Characteristics of Morphine

<u>Frequency cm<sup>-1</sup></u>	<u>Assignment</u>
3480, 3350	Free OH
3210	Bonded OH (Bonded)
2940, 2920	CH (stretch)
2840	H <sub>3</sub> C - NH <sup>+</sup>
1650	C <sub>7</sub> =C <sub>8</sub> (alkene)
1605	C=C (aromatic)
1250, 1090	C <sub>4</sub> -O-C <sub>5</sub>
760	Monosubstituted aromatics

The IR of morphine exhibited the following other characteristic absorption bands:-

2825, 1635, 1475, 1448, 1370, 1360, 1330, 1310, 1280, 1265, 1200, 1170, 1145, 1120, 1045, 1030, 980, 960, 945, 920, 890, 835, 805, 790, 720, 710, 675, 658, 610, 586, 570 and 525 cm<sup>-1</sup>.

Other IR data for morphine and morphine hydrochloride have been reported (3,26,27).

### 2.9.3 Nuclear Magnetic Resonance Spectra

#### 2.9.3.1 Proton Spectra (PMR)

The PMR spectra of morphine hydrochloride in D<sub>2</sub>O (Fig.7) and morphine in DMSO-D<sub>6</sub> (Fig.8) were recorded on a Varian XL 200, 200 MHz and on a Varian T60A60 MHz NMR spectrometers respectively using TMS (tetramethylsilane as an internal reference in both. The following structural assignments have been made (Table 6).

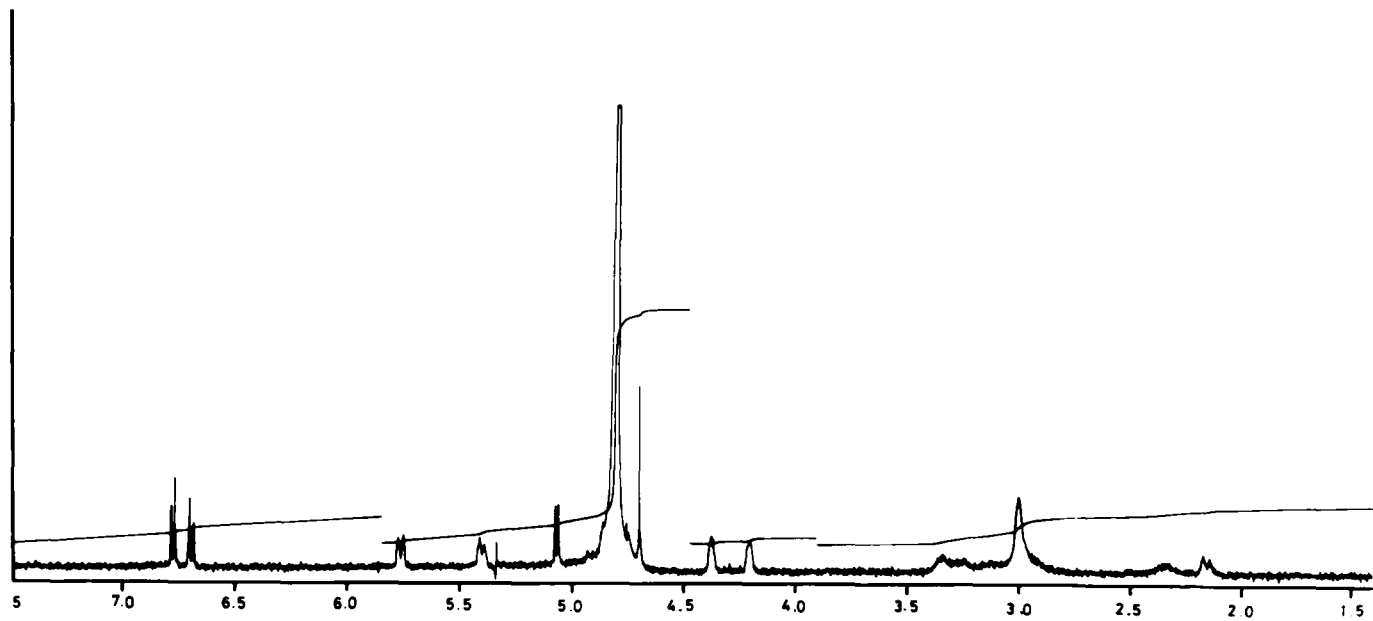


FIG. 7 : THE  $^1\text{H}$ -NMR SPECTRUM OF MORPHINE HYDROCHLORIDE IN  $\text{D}_2\text{O}$ .

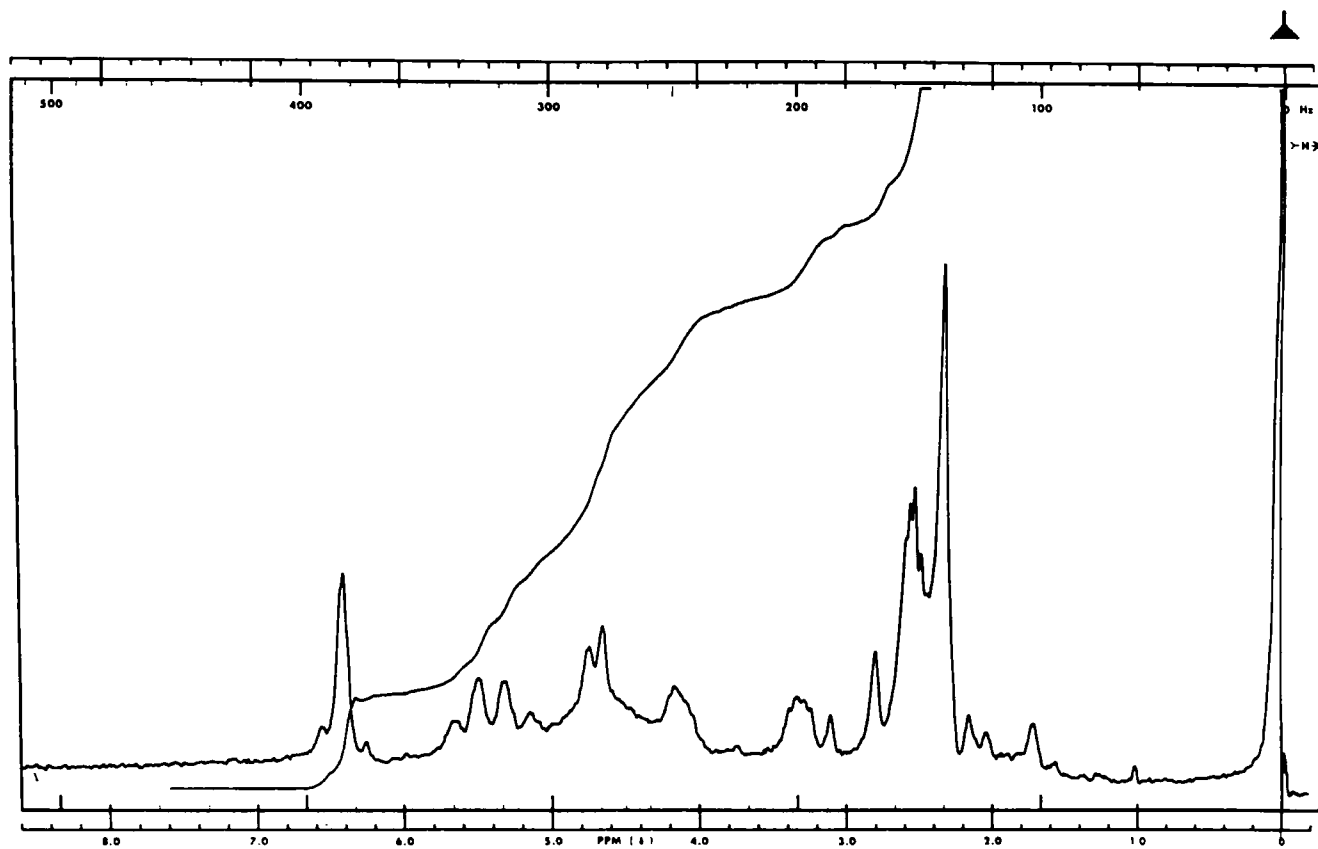


FIG. 8 : THE  $^1\text{H}$  NMR SPECTRUM OF MORPHINE IN  $\text{DMSO}-d_6$ .



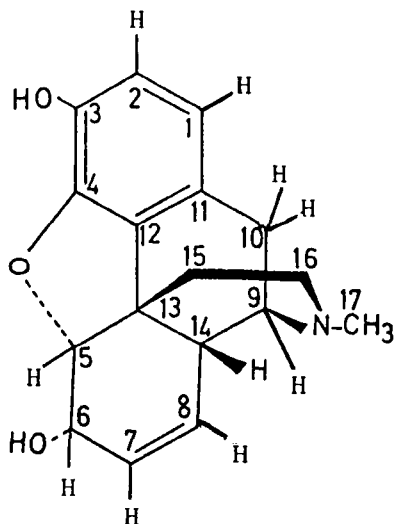
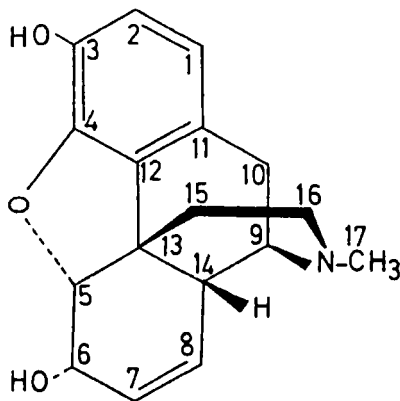


Table 6. PMR Characteristics of Morphine

Group	Chemical Shift (ppm)	
	Morphine HCl	Morphine
H <sub>2</sub> (aromatic)	6.76 (d)	6.42 (ABq)
H <sub>1</sub> (aromatic)	6.68 (d)	
H <sub>7</sub>	5.75 (d)	
H <sub>8</sub>	5.40 (d)	
H <sub>5</sub>	5.06 (d)	4.63 (d)
(J <sub>5,6</sub> 2Hz)		
H <sub>9</sub>	4.37 (m)	
H <sub>14</sub>	4.20 (d)	
N-CH <sub>3</sub>	3.00 (s)	2.30 (s)
2H <sub>10</sub>	2.16	

s = singlet, d = doublet, m = multiplet,  
q = quartet.

Other <sup>1</sup>H-NMR data for morphine and its salts have been reported (3,28-31).

2.9.3.2  $^{13}\text{C}$ -NMR

The  $^{13}\text{C}$ -NMR completely decoupled and off-resonance spectra of morphine and morphine hydrochloride were recorded over 5000 Hz using a Joel FX-100 NMR spectrometer. Morphine was dissolved in  $\text{DMSO-d}_6$  using 10 mm sample tube and tetramethylsilane as internal reference standard at  $20^\circ$ . Morphine hydrochloride was dissolved in  $\text{D}_2\text{O}$  using 10 mm sample tube (conc. 80 mg/1 ml) and dioxane as an internal reference standard at ambient. The spectra of morphine hydrochloride are presented in Fig. 9 and Fig. 10. The carbon chemical shifts were assigned on the basis of the additivity principles and off-resonance splitting pattern. Assignment of all 17C of morphine (Table 7) are consistent with those reported by Carroll et al (28).

Table 7. Carbon Chemical Shifts of Morphine

Carbon No	Chemical Shift [ppm]		Carbon No	Chemical Shift [ppm]	
	Morphine	Morphine HCl		Morphine	Morphine HCl
C <sub>1</sub>	118.30	121.24 (d)	C <sub>4</sub>	146.10	146.36 (s)
C <sub>2</sub>	116.13	118.48 (d)	C <sub>5</sub>	91.24	91.12 (d)
C <sub>3</sub>	138.20	138.85 (s)	C <sub>6</sub>	66.11	66.58 (d)

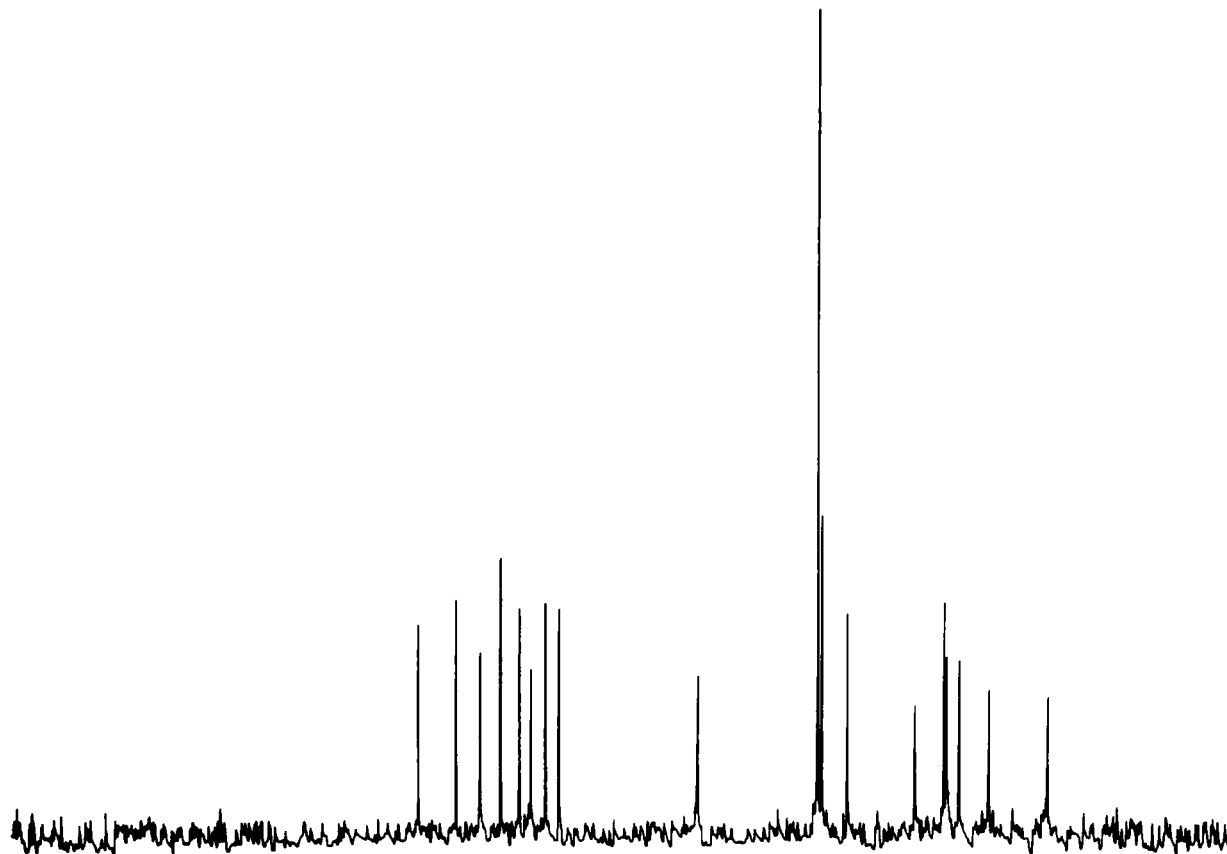


FIG. 9 : THE  $^{13}\text{C}$ -NMR COMPLETELY DECOUPLED SPECTRUM OF MORPHINE HYDROCHLORIDE.

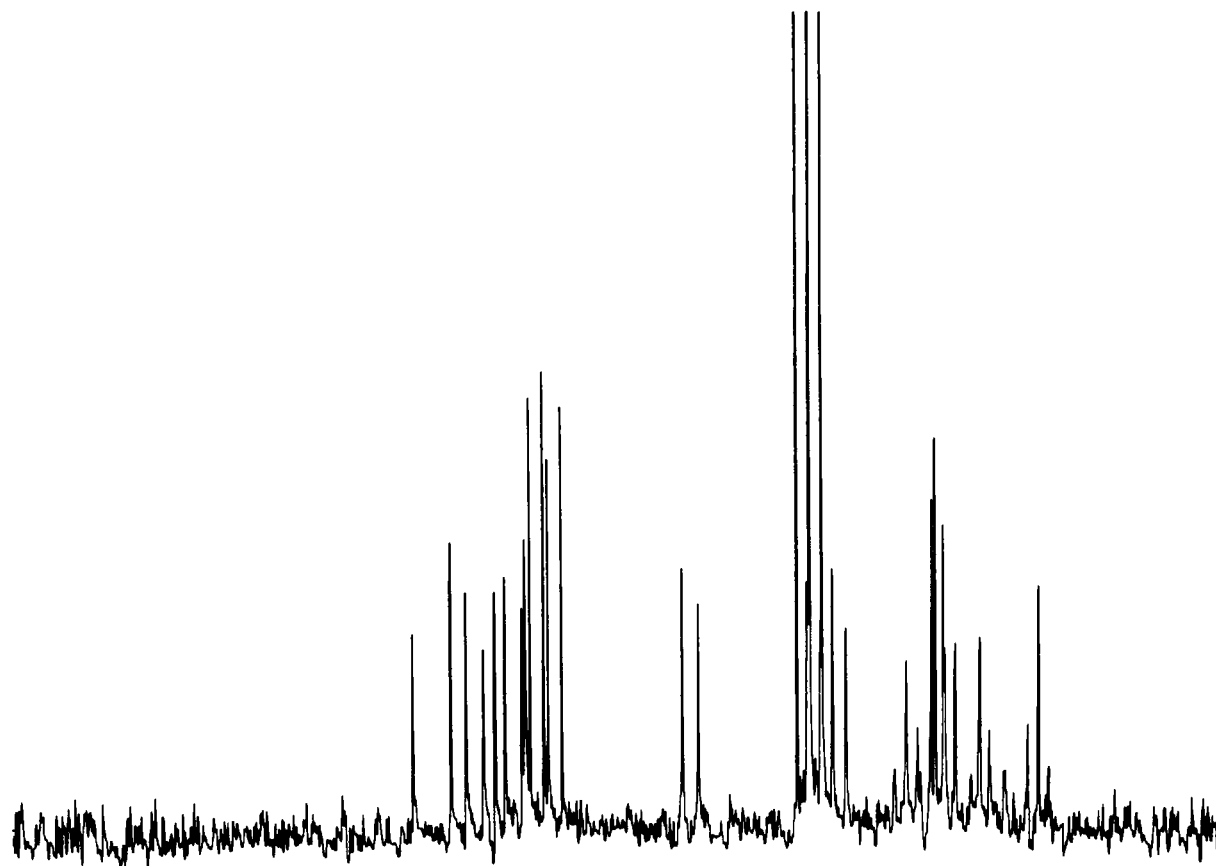


FIG.10 : THE  $^{13}\text{C}$ -NMR OFF RESONANCE SPECTRUM OF MORPHINE HYDROCHLORIDE.

Carbon No	Chemical Shift [ppm] Morphine	Chemical Shift [ppm] Morphine HCl	Carbon No	Chemical Shift [ppm] Morphine	Chemical Shift [ppm] Morphine HCl
C <sub>7</sub>	133.16	134.03 (d)	C <sub>13</sub>	42.74	42.39 (s)
C <sub>8</sub>	128.22	126.40 (d)	C <sub>14</sub>	41.04	41.86 (d)
C <sub>9</sub>	57.83	61.53 (d)	C <sub>15</sub>	35.94	33.47 (t)
C <sub>10</sub>	19.90	21.84 (t)	C <sub>16</sub>	45.79	48.09 (t)
C <sub>11</sub>	125.29	124.17 (s)	C <sub>17</sub> (NCH <sub>3</sub> )	39.39	39.34 (q)
C <sub>12</sub>	130.75	130.10 (s)			

s = singlet; d = doublet; t = triplet; q = quartet

Other <sup>13</sup>C-NMR data for morphine have also been reported (28,32,33).

#### 2.9.4 Mass Spectrum

The mass spectrum of morphine is presented in Fig. 11. This was obtained by electron impact ionization on a Ribermag R-10-10 mass spectrometer equipped with direct inlet probe at 270°. The electron energy was 70 eV. The spectrum scanned to mass 350 amu. The spectrum (Fig. 11) shows a molecular ion peak M<sup>+</sup> at m/e 285 with a relative intensity 100%. The most prominent fragments and their relative intensities are listed in table 8.

Table 8 : The Mass Fragments of Morphine

m/e	Relative Intensity %	m/e	Relative Intensity %
285	100.0 (base)	128	31
286	26.7 M <sup>+1</sup>	127	27.8
284	17.2 M <sup>-1</sup>	124	43.3
268	13.3	116	16.6
216	11.1	115	74
215	33.3	109	22.2
174	18.9	103	16.7
171	11.1	94	26.7
162	57.8	91	21.7
161	11.0	82	22.2
153	13.3	81	42.7
152	22.2	77	32.2
131	24.4	70	46.6
129	14.4	65	22.2

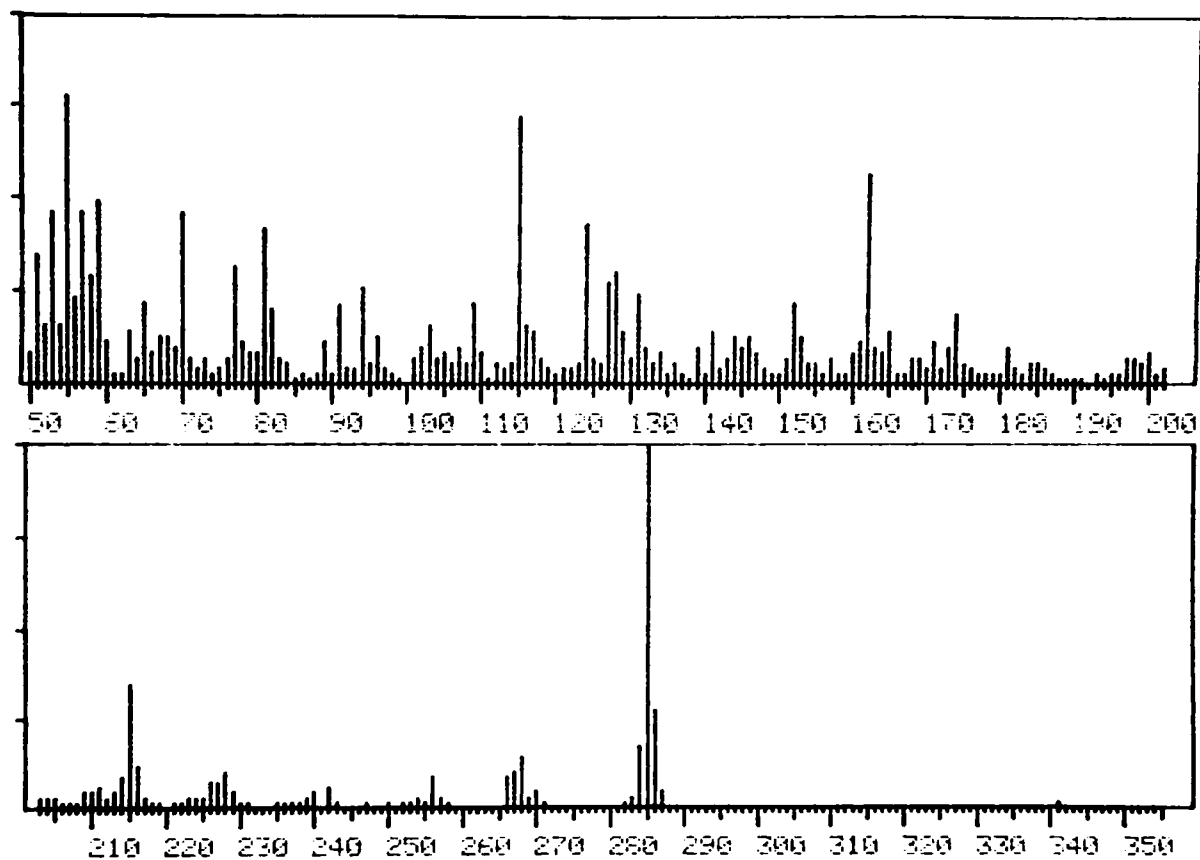


FIG.11 : THE MASS SPECTRUM OF MORPHINE.

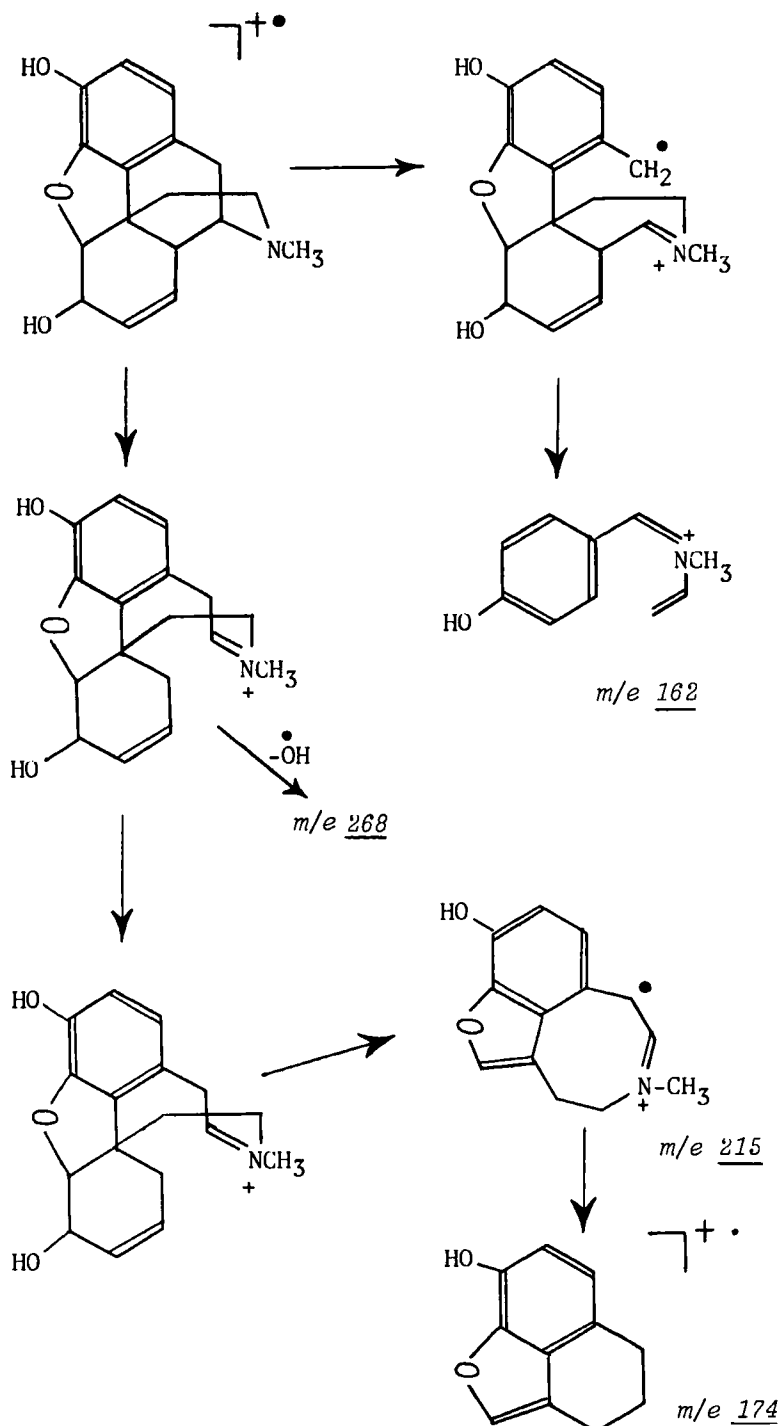
m/e	Relative Intensity %	m/e	Relative Intensity %
60	12.2	55	80
59	50	54	16.6
58	28.9	53	47.8
57	46.6	52	15.5
56	23.3	51	35.6

Mass fragmentation of morphine is presented in Fig. 12 (34).

Mass spectrometry can be effectively used to distinguish between compounds with rings B/C cis fused and those with rings B/C transfused. N-methyl compounds in the B/C cis series (as natural morphine), show an intense peak at m/e 59, which is absent in compounds in the B/C trans series. Thus morphine shows an intense peak at m/e 59 with relative intensity of 50% (Table 8).

Other mass data for morphine have also been reported (34-38).

Fig. 12. Mass Fragmentation of Morphine





### 3. Isolation of Morphine

Morphine occurs in varying amounts from 5 to 24% in opium. Official opium (of the U.S.P. and the B.P.) should contain not less *than* 9.5% of anhydrous morphine (11,17). Opium is the dried latex (milky exudate) obtained by incision of the unripe capsules of the poppy plant, *Papaver somniferum*, Family *Papaveraceae*.

Morphine is the most important alkaloid of opium, and it is the first plant base to be isolated and recognized. It was first isolated by a German apothecary, Sertürner in 1806 (39). Since its isolation and characterization, morphine became an important drug. It is a typical narcotic analgesic drug to which all other narcotic analgesics are measured.

Morphine is only obtained from opium (40) and several processes are in use. In nearly all of these processes morphine and most of the other opium alkaloids are extracted with water alone or with slightly acid water (41).

#### Procedure

Powdered opium is extracted either with warm water or slightly acid water to complete exhaustion. The extract is concentrated under vacuum, treated with a solution of calcium chloride (1:1), left for 48 hours and then filtered. The filtrate containing the hydrochlorides of the alkaloids is concentrated when morphine and codeine hydrochlorides deposit in the form of double compound known as "Gregory salt". This salt is dissolved in warm water and neutralized with dilute ammonia to phenolphthalein end point (pH 9). Morphine precipitates, while codeine remains in the solution as ammonium-codeine chloride. The crude morphine so obtained is purified by repeated crystallization from hot alcohol.

In another process, the concentrated water extract is mixed with alcohol and made strongly alkaline with ammonia, the morphine being slightly soluble in dilute alcohol, separates out while the greater of the other alkaloids remain in solution. Morphine is collected and purified by repeated crystallization as the sulfate or as the hydrochloride and reprecipitation with ammonia in the presence of alcohol (41).

#### Formation of Morphine hydrochloride and Morphine sulfate

These salts can be prepared by neutralizing a hot aqueous suspension of morphine either with dilute hydrochloride acid or with dilute sulfuric acid. The resulting solution is concentrated to crystallization (42).

Achor and Geiling ( 43 ) developed a new extraction process for the isolation of semi-micro quantities of morphine from opium by using ion exchange chromatography. They found that Nalcite SAR was the best quaternary base resin for the separation of morphine, Leete ( 44 ) has simplified this method as follows:-

Fresh poppies (57g) were macerated in a blender with a (1:1) mixture of 1-butanol-benzene (200 ml) and 10% aqueous sodium carbonate (40 ml). After standing for 48 hours, the mixture was filtered and the marc washed with a further 50 ml of the organic solvent. The combined organic layer was washed several times with 0.5 N sulfuric acid (150 ml). The acid extract was made alkaline by the addition of 2% aqueous solution of potassium hydroxide saturated with barium hydroxide. The barium sulfate was filtered off and the filtrate was added to the top of a column (20 x 1.5 cm.) of Nalcite SAR ion exchange resin in the hydroxide form. The column was then washed with 50% aqueous methanol (200 ml). The morphine was eluted from the column with 0.1 N hydrochloric acid. Morphine appeared in the eluate as soon as the *pH* decreased to 2.0. When 50 ml of such eluate had been collected it was evaporated to dryness in vacuo. The residue was dissolved in a minimum of water, filtered and made alkaline with a drop of sodium carbonate solution. The morphine crystallized out and was dried at 100°.

- Other methods of isolation have been reported (45-47).

Morphine can be commercially separated from opium by counter-current extraction technique at pH 9.1 to 9.3 with benzene-butanol (1:1) or with cresol-benzene-butanol (10:45:45). It is then extracted from the organic solvents by either dilute sulfuric acid or dilute phosphoric acid and precipitated from the acid solution by ammonia solution (45).

#### 4. Synthesis of Morphine

*The first total synthesis of morphine was achieved in 1956 by Gates and Tschudi (2), although Gates started the synthesis of morphine intermediates as early as 1948 (48,49). The following is the scheme which was carried out by Gates:*

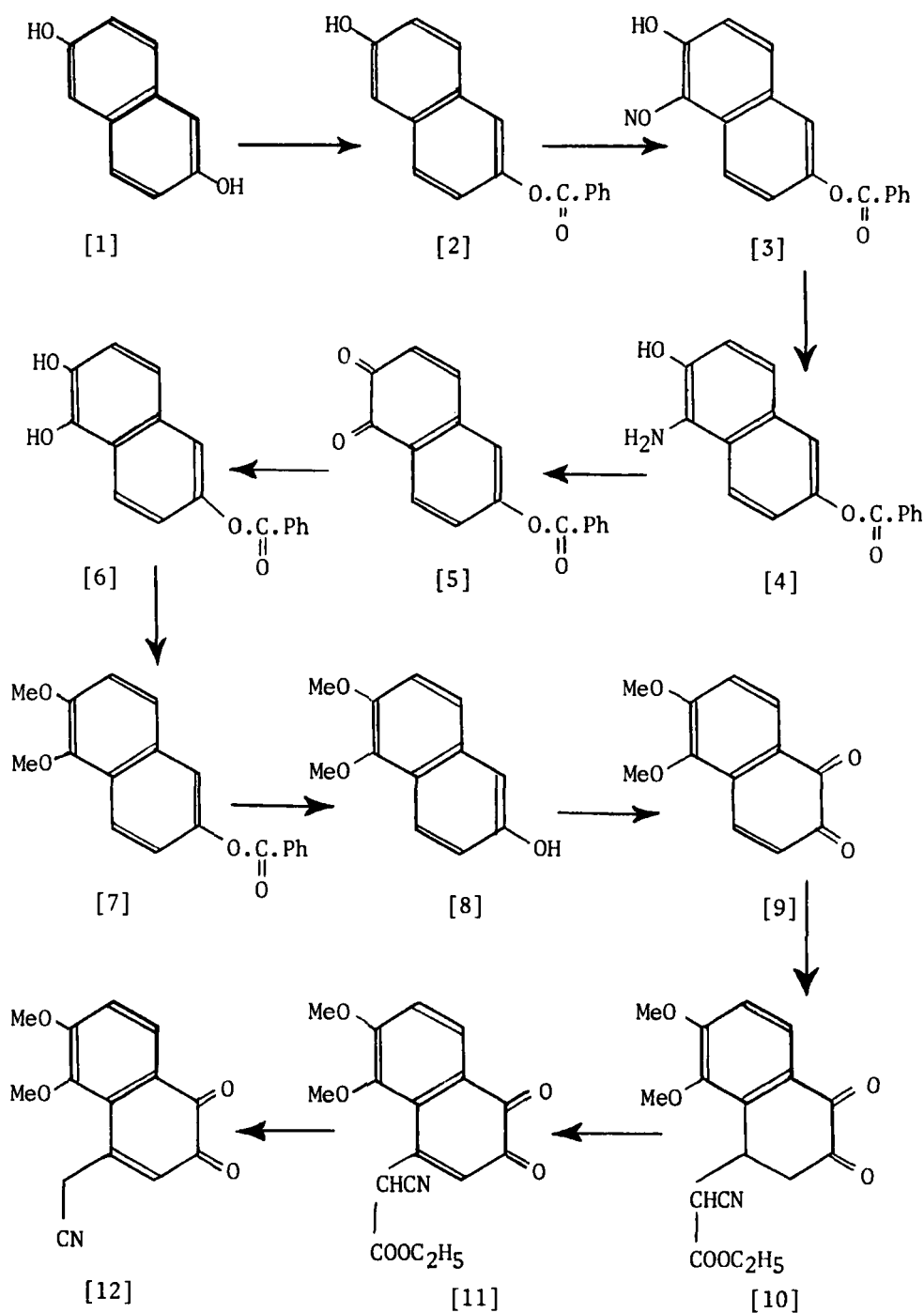
2,6-Dihydroxynaphthalene [1] was benzoylated with benzoyl chloride and pyridine to give 2-hydroxynaphthalene-6-monobenzoate [2]. Nitrosation of [2] with sodium nitrite in the presence of aqueous acetic acid yielded the 1-nitroso-2-naphthol-6-monobenzoate [3], which was reduced to the 1-amino-2-naphthol-6-monobenzoate [4]. The latter was oxidized with ferric chloride to give 6-benzoyloxy-1,2-naphthoquinone [5] which was readily reduced by sulfur dioxide to provide the 6-benzoyloxy-1,2-naphtho hydroquinone [6]. The hydroxyl groups of [6] were methylated with dimethyl sulfate and alkali to give 5,6-dimethoxy-2-naphthol benzoate [7]. The benzoyl group of [7] was removed by hydrolysis to afford the 5,6-dimethoxy-2-naphthol [8]. The latter was subjected to the previous sequence of reactions (nitrosation and conversion to the 1,2-quinone through the aminonaphthol) to give 5,6-dimethoxy-1,2-naphthoquinone [9]. This was condensed with ethyl cyanoacetate (Michael condensation) to provide the condensate [10]. The latter was oxidized under mild conditions to produce ethyl (5,6-dimethoxy-1,2-naphthoquinonyl-4)-cyanoacetate [11].

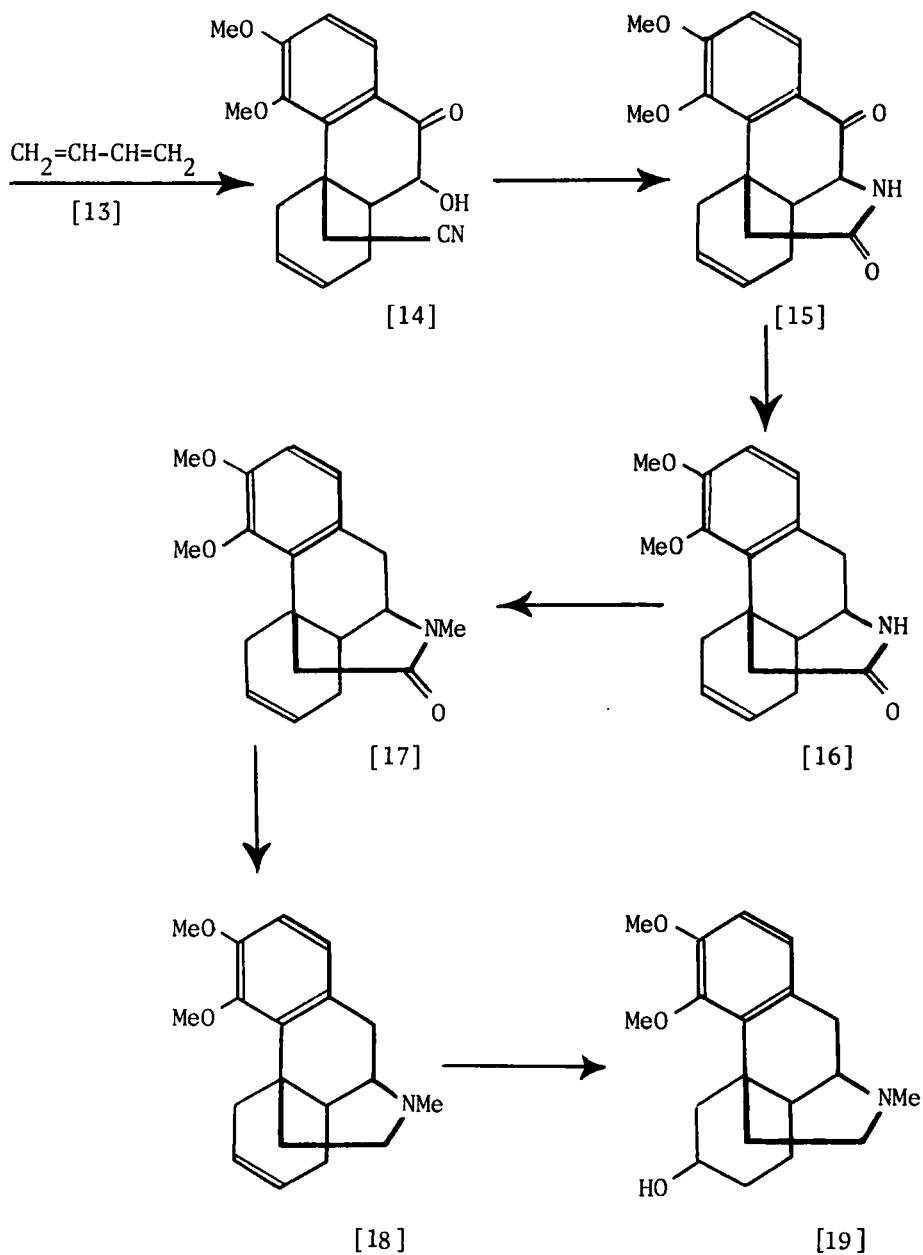
Selective hydrolysis of [11] gave the salt  $\alpha$ -cyanoacid which on acidification, readily underwent decarboxylation to yield 5,6-dimethoxy-4-cyanomethyl-1,2-naphthoquinone [12]. This key intermediate underwent the Diels-Alder reaction on treatment with butadiene [13] to give the 3,4-dimethoxy-9,10-dioxo-13-cyanomethyl-5,8,9,10,13,14-hexahydrophenanthrene [14].

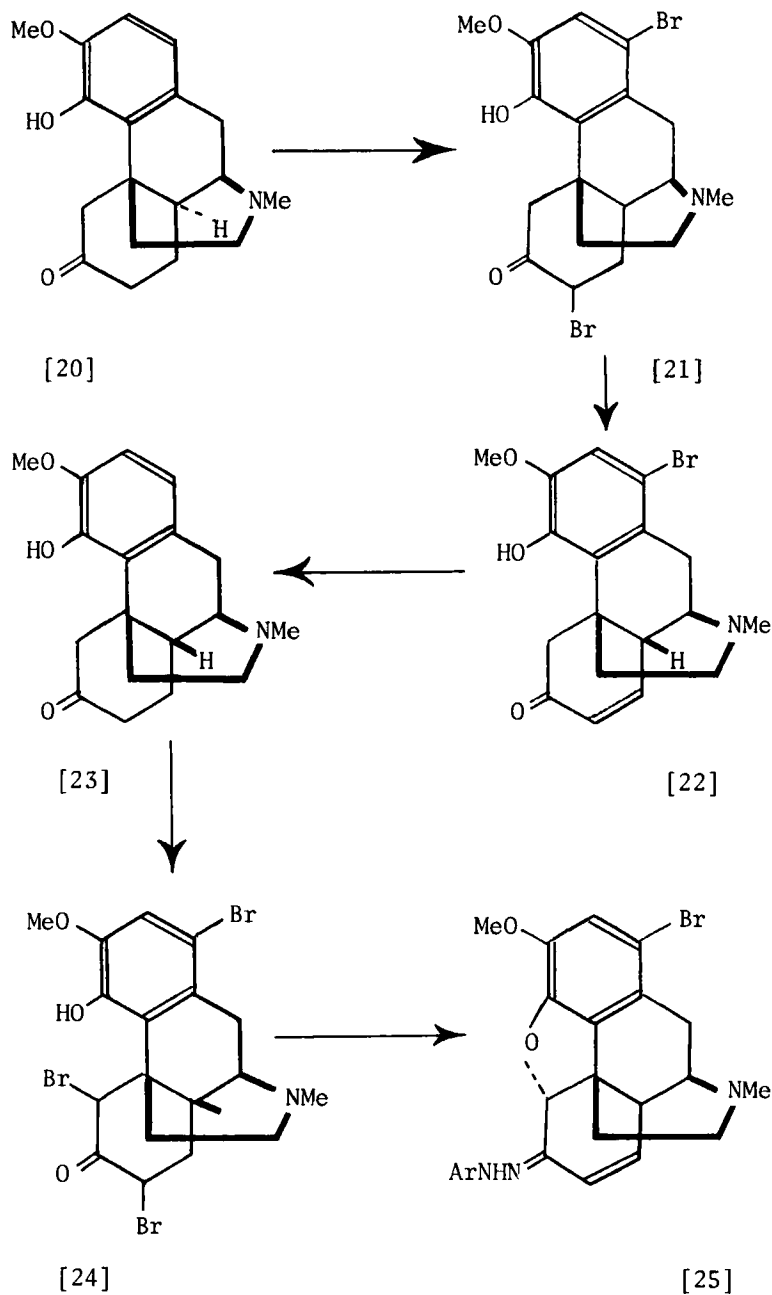
Adduct [14] was reduced with copper chromite under mild conditions, underwent ring closure to the ketolactam [15]. Reduction of [15] (Wolf-Kischner method) gave [16] which was N-methylated with sodium hydride and methyl iodide to the N-methyl derivative [17]. The latter was reduced with lithium aluminium hydride to  $(\pm)$ - $\beta$ - $\Delta^6$ -dihydrodesoxycodine methylether [18]. Since [18] can be obtained from natural sources (by degradation of thebaine), further synthetic steps could be carried out on the natural compound. Synthetic [18] however, was resolved with dibenzoyl-L(+)-tartaric acid and the (+)-base was also employed in the synthesis.

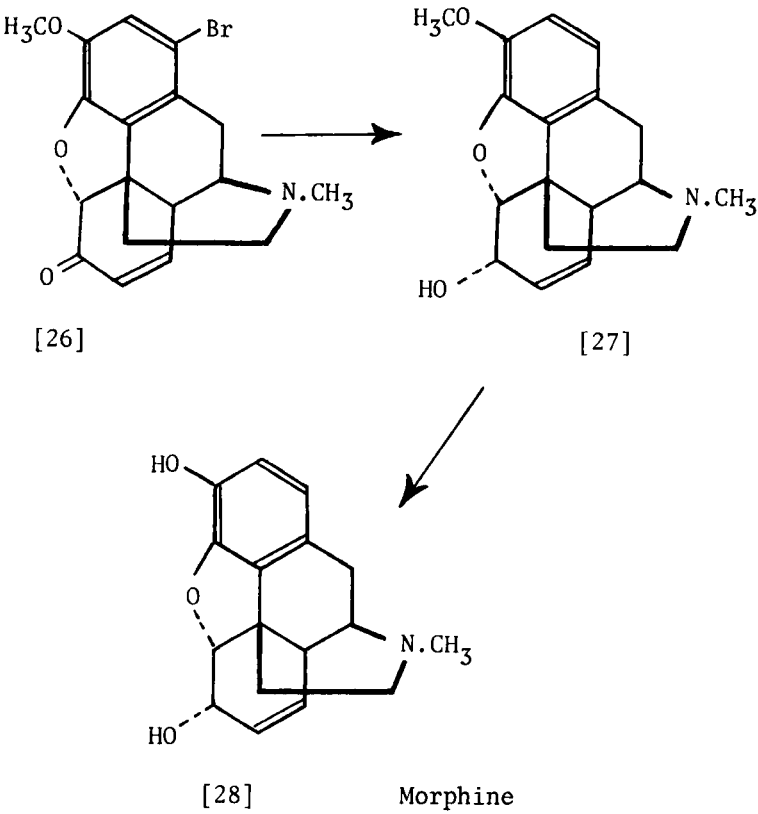
The optically active [18] was hydrated with dilute sulfuric acid to yield  $\beta$ -dihydrothebainol methylether [19].

SCHEME I: THE FIRST TOTAL SYNTHESIS OF MORPHINE  
(*Gates and Tschudi*)









This was demethylated with a mixture of diethylene glycol, potassium hydroxide and hydrazine hydrate to give  $\beta$ -dihydrothebainol which was oxidized by Oppenauer oxidation to provide the  $\beta$ -dihydrothebainone [20]. The latter was brominated to the dibromo-derivative [21] and this was treated with 2,4-dinitrophenylhydrazine to give the mono-bromo- $\alpha$ ,  $\beta$ -unsaturated ketone dinitrophenylhydrazone (which has the *cis* configuration). This was easily cleaved with acetone and hydrochloric acid to the 1-bromo-thebainone [22]. Reduction of [22] over palladium gave the dihydrothebainone [23]. The latter was brominated to produce the tribromoderivative [24] which was treated with 2,4-dinitrophenylhydrazine when oxide ring closure occurred to give the hydrazone derivative [25]. Cleavage of [25] with acetone and hydrochloric acid gave the 1-bromo-codeinone [26]. Reduction of [26] with lithium aluminium hydride caused the removal of the bromine atom and the formation of the correct alcohol epimer (axial), Codeine [27]. The latter was demethylated with hydrosulfite in pyridine to render morphine [28].

*Gates and Tschudi's total synthesis of morphine is presented in Scheme I.*

A second synthesis of morphine is adopted by Elad and Ginsburg ( 50 ) who synthesized (-)-dihydrothebainone [23] from the starting material 2-(2,3-dimethoxyphenyl)-2-cyclohexanone which was prepared from the readily available 1-(2,3-dimethoxyphenyl)-cyclohexane. This constitutes a formal total synthesis of morphine since [23] is converted into morphine by Gates and Tschudi's method of synthesis.

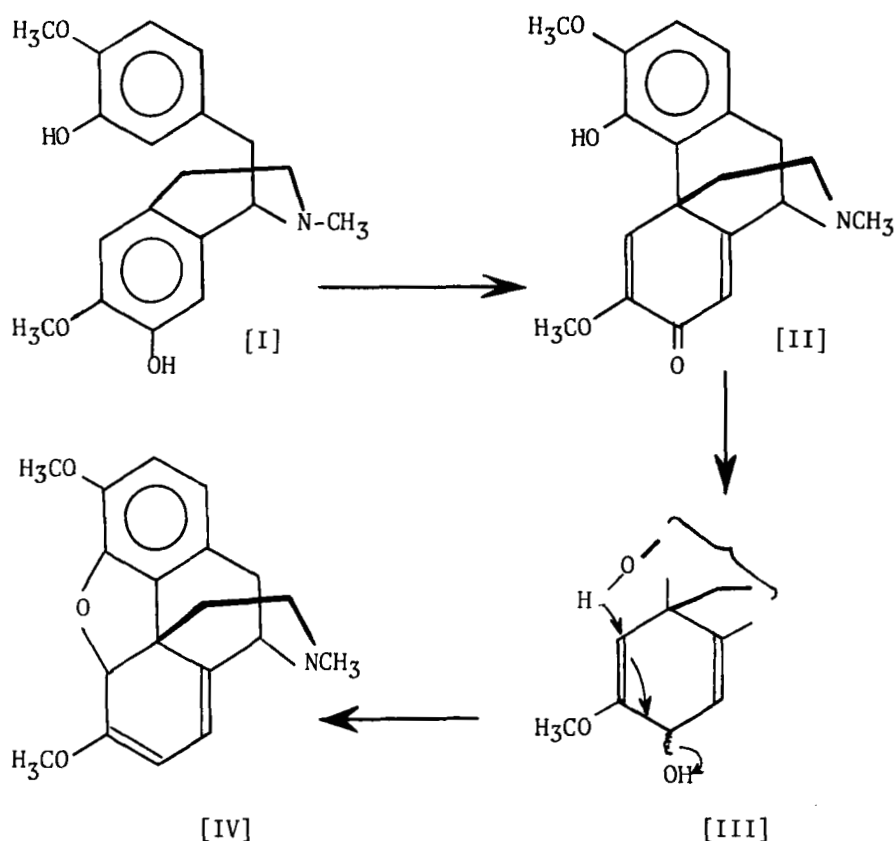
A third synthesis of morphine was carried out by Barton et al ( 51 ) during their investigation of the biosynthesis of morphine through dienone. Benzylisoquinoline [I] which has been synthesized earlier by several authors ( 51-54 ) was oxidized with manganese dioxide to give the dienone [II]. This was reduced with sodium borohydride to afford via the allylic alcohol [III], thebaine [IV]. The latter was demethylated at C<sub>6</sub> to codeine which also underwent demethylation at C<sub>3</sub> to morphine. *This synthesis is presented in Scheme II.*

The fourth formal total synthesis of morphine has been reported by Morrison et al (55).

The benzyltetrahydroisoquinoline [I] (which is readily available from *m*-methoxyphenethylamine and 3,4-dimethylphenylacetic acid via amide formation, Bischler-Napieralski cyclization, reduction and methylation).

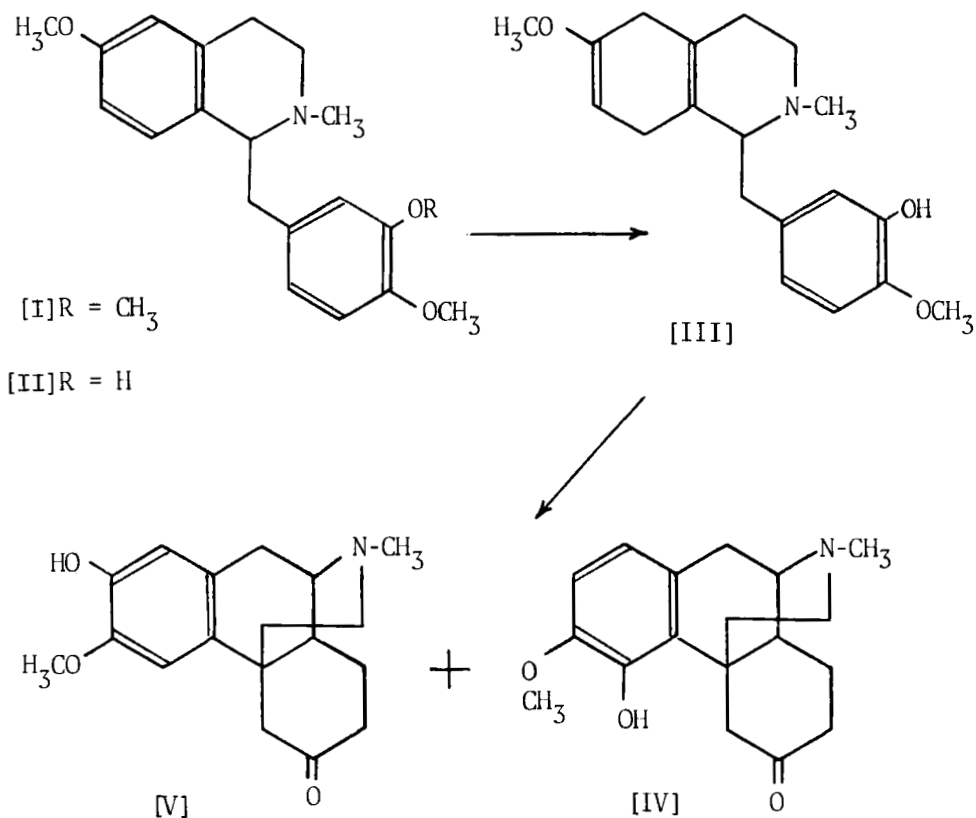


## SCHEME II: THE THIRD SYNTHESIS OF MORPHINE



The Birch reduction of [I] with sodium and *t*-butanol in liquid ammonia results in cleavage of the 3-methoxy group of the benzyl function and reduction of the isoquinoline moiety to give [II]. Refluxing of [II] with 10% hydrochloric acid brings about hydrolysis of the enol ether, conjugation of the double bond and cyclization to ( $\pm$ )-dihydrothebaine [III] in 3% yield and its positional isomer [IV] in 37% yield. Since conversion of [III] to morphine has already been accomplished by Gates and Tschudi's method (2). This method represents a total synthesis of morphine. Scheme III represents this synthesis.

## SCHEME III: THE FOURTH SYNTHESIS OF MORPHINE



Other methods for the synthesis of (-)-morphine (56,57) and (+)-morphine have been reported (58-60).

## 5. Biosynthesis of Morphine

Postulation of the biosynthetic pathway of opium alkaloids started as early as 1910 with the suggestion of Winterstein and Trier ( 61 ) that the benzylisoquinoline alkaloids are built up in nature from two units of 3,4-dihydroxyphenylalanine (DOPA).

Gulland and Robinson (1) proposed that morphine arises in the plant from a suitable benzylisoquinoline precursor (norlaudanosoline [3]) by rotation of this precursor followed by oxidative ring closure. The validity of such schemes remained untested until the advent of radiochemical techniques, when in 1958-1960 experiments with labelled tyrosine, administered to poppy capsules, demonstrated that the two molecules of DOPA were incorporated into the morphine molecule, in full accord with Robinson's theory. *(These two molecules of DOPA gave rise to 3,4-dihydroxyphenylethylamine and 3,4-dihydroxyphenylpyruvic acid by undergoing decarboxylation and oxidative deamination respectively).*

Battersby and Co-workers in 1958 and 1960 (62,63) and and Leete (44,64) have established that when 2-<sup>14</sup>C-tyrosine was fed into poppy plants, radioactive thebaine [9], codeine [12] and morphine were obtained. These alkaloids upon degradation, were shown to be labelled equally and specifically at C<sub>9</sub> and C<sub>16</sub> as expected. By feeding 1-<sup>14</sup>C-dopamine, Battersby and Francis ( 65 ) found that only C<sub>16</sub> of these alkaloids were labelled but not C<sub>9</sub>. Upon biological condensation of one unit of 3,4-dihydroxyphenylethylamine with one unit of 3,4-dihydroxyphenylpyruvic acid gives rise to a benzylisoquinoline system i.e. norlaudanosoline carboxylic acid [1], biological decarboxylation occurs to produce 1,2-dehydronorlaudanosoline [2] which upon biological reduction renders norlaudanosoline [3]. Further, the intermediate stage was confirmed by the demonstration that [1-<sup>14</sup>C]-norlaudanosoline acted as a more efficient precursor for morphine than did tyrosine and afforded a product labelled as required by the theory (66).

Battersby et al ( 67 ) have further found that (-)-reticuline [6] is the most efficient precursor to morphine *((-)-reticuline was found to be as a natural alkaloid in opium) (68).*

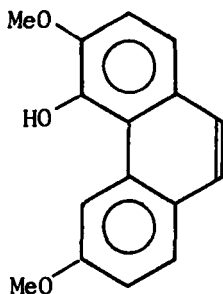
Barton et al ( 69 ) established that salutaridine [7] *(which do exist in trace amounts in opium) is formed by phenolic oxidative coupling of (-)-reticuline [6].*

It has been suggested that thebaine [9] is the first phenanthrene alkaloid to be formed from salutaridine [7] through salutaridinol [8] and [9] is biologically converted to codeine [12] and then to morphine (18,70). Rapaport (71,72) has confirmed this experimentally by exposing poppy plants to  $^{14}\text{C}\text{O}_2$  for a varying lengths of time and by injecting labelled alkaloids into the living poppy plants. He further showed that thebaine is irreversibly converted into codeine and then to morphine. Battersby (73) has also independently reached to the same conclusion. The existence of codeinone [11] as an intermediate between thebaine [9] and codeine [12] was confirmed (74).

In recent years many details of the biosynthetic pathway of morphine have been filled in, particularly neopinone [10] (75,76).

*The biosynthesis of morphine is illustrated in scheme IV. (75).*

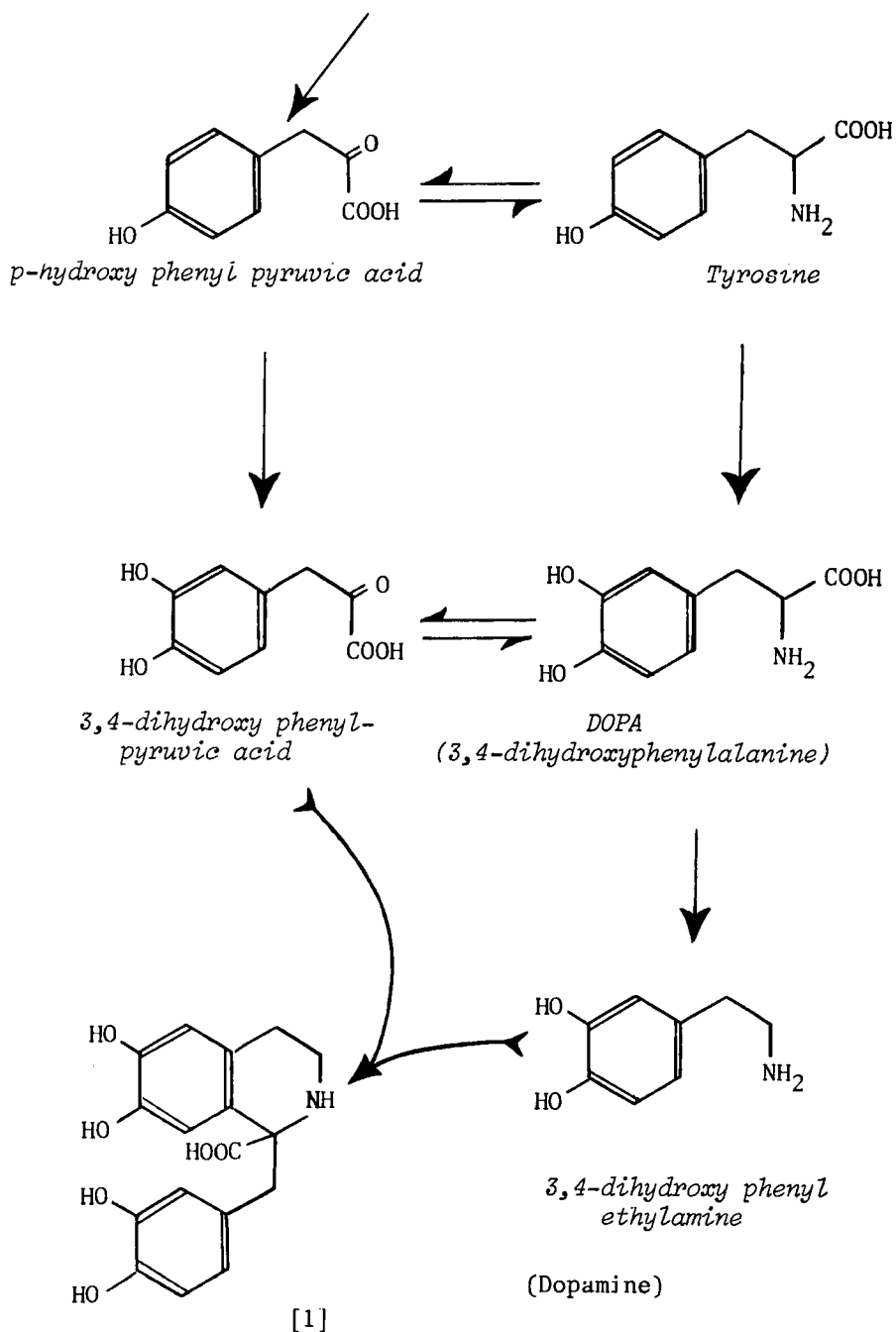
Although morphine has been generally considered to be an end-product of alkaloid metabolism in *Papaversomniferum*, Fairbrain and co-workers (77-79) as a result of feeding experiments involving radioactive morphine, have suggested that it is readily metabolized into a series of compounds. Some of these compounds are alkaloid-like and others are bound forms of the alkaloids which are present in the pericarp, stored in the seeds and then broken down into smaller alkaloid-like substances on germination; codeine has been isolated as one of these break down products. Other researchers have also shown that  $^{14}\text{C}$ -morphine upon feeding into poppy plant is degraded to non-alkaloidal substances such as thebaol which is elaborated directly by the plant from thebaine (80,81).



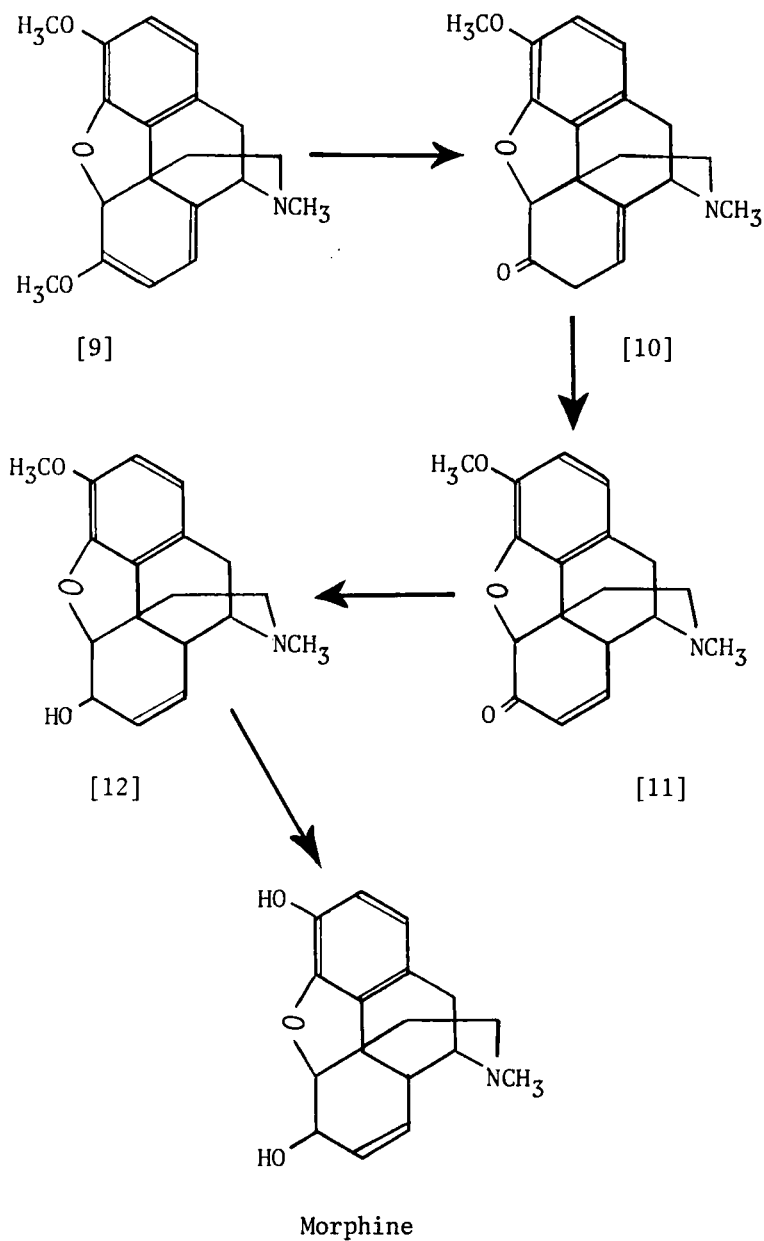
Thebaol

## SCHEME IV: THE BIOSYNTHESIS OF MORPHINE

## Shikimic Acid Pathway







## 6. Drug Absorption, Distribution, Metabolism and Excretion

### 6.1 Drug Absorption

#### 6.1.1 Orally

Morphine is readily absorbed from the gastrointestinal tract. It is also absorbed from the nasal mucosa and the lung (as when heroin is snuffed or as opium is smoked) (82).

Upon absorption, oral morphine is rapidly conjugated with D-glucuronic acid in the cells of intestinal mucosa and in the liver that significant levels of free morphine are not found in either the plasma nor the urine, whereas the level of conjugated morphine are high (82,83).

N-demethylation of morphine is greater after oral than after parenteral administration (83).

#### 6.1.2 Parenterally

Morphine is readily absorbed after parenteral administration (83,84).

Morphine is rapidly absorbed after intramuscular (IM) and subcutaneous (SC) injection, Producing plasma levels of free morphine, from 15 minutes to 3 hours, which are significantly higher than levels after intravenous (IV) administration (83).

Intravenous morphine, while initially higher, undergoes more rapid distribution, metabolism and excretion (83).

### 6.2 Distribution and Fate

When therapeutic concentrations of morphine are present in plasma, 34 to 37.5% (about one-third) of the drug is protein bound (82,85). This amount is reduced in patients with renal failure (85). Free morphine rapidly leaves the blood to accumulate in parenchymatous tissues of the kidney, lung, liver and spleen. It is also present in the skeletal muscle, but in lower concentrations than the parenchymatous tissues (82).



### 6.3 Drug Metabolism

The major pathway for the detoxication of morphine is conjugation either with D-glucuronic acid in the liver to produce several glucuronides, such as *morphine 3-monoglucuronide*; *morphine 6-monoglucuronide* and *morphine 3,6-diglucuronide* (82,86-94) or with sulfuric acid to give *morphine 3-ethereal sulfate* (88-94).

Other metabolic pathways include:-

- a) N-demethylation of morphine to yield *normorphine* (94-97), which is then conjugated to give *normorphine 6-glucuronide* (83,94,98).
- b) O-C<sub>3</sub>methylation of morphine to form *codeine* (99-102) which is also undergone N-demethylation to give *norcodeine* (102).
- c) Oxidation of morphine to produce *morphine 2,3-quinone* (94,103), *Dihydromorphinone* (104).

The metabolism of morphine in man and animals is presented in Fig. 13 and the structures of prominent morphine metabolites are shown in Fig. 14.

### 6.4 Excretion

Morphine is excreted in the urine within the first 24 hours as follows:-

- a) 8.5 to 12% of the dose as free unchanged morphine (83,105).
- b) 11 to 60% of the dose as morphine metabolites (83,105).

It was also found that 90% of the total excretion of morphine takes place during the first day (24 hours). The major route of elimination of the metabolites is by *glomerular filtration* (82).

About 7 to 10% of administered morphine eventually appears in the feces, and this comes almost exclusively from the bile as conjugated morphine (82).

Enterohepatic circulation of morphine and morphine glucuronides occurs, which probably accounts for the presence of morphine in the urine for several days after the last dose (82).

Trace amounts of morphine are excreted into breast milk and these amounts are insignificant following clinical use, but in addicts, drug concentrations may become significant (106).

FIG. 13: THE METABOLISM OF MORPHINE

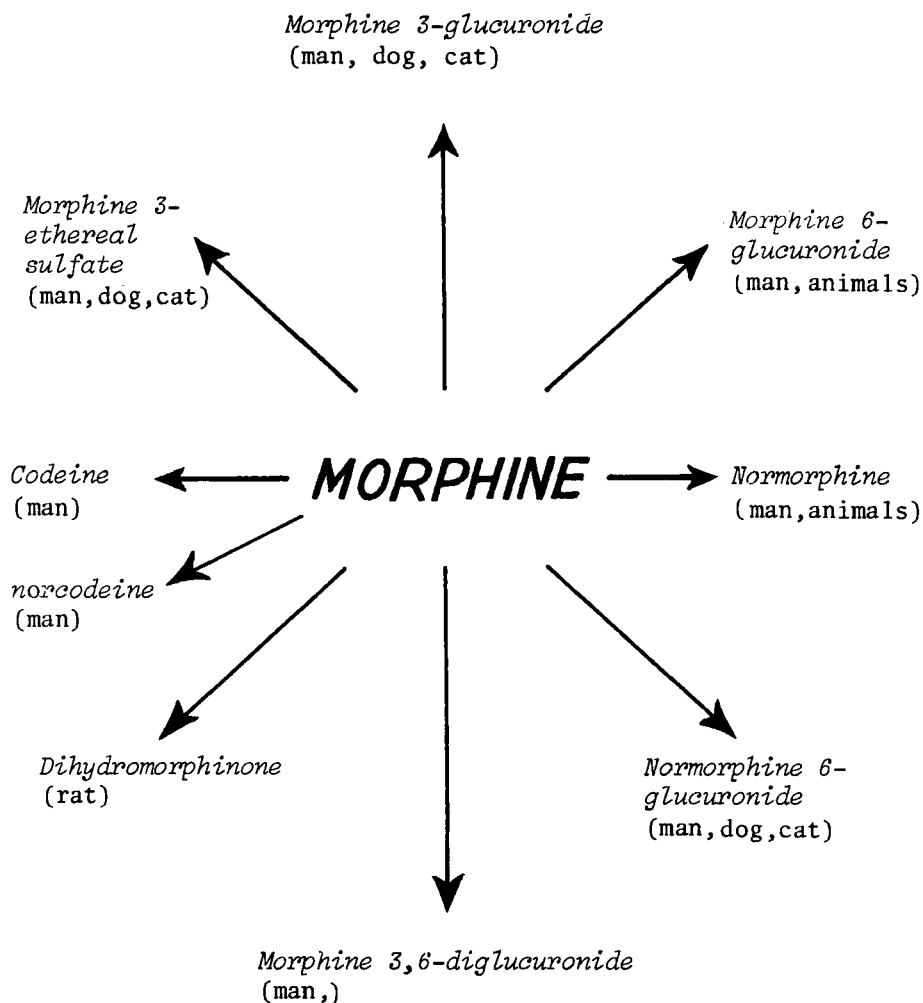
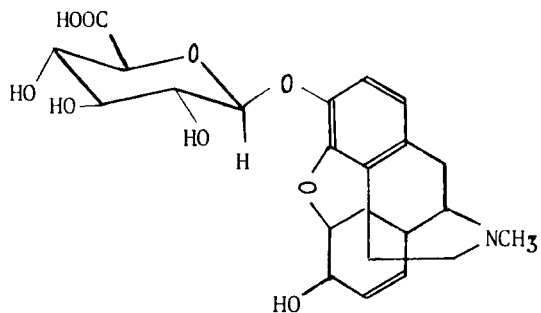
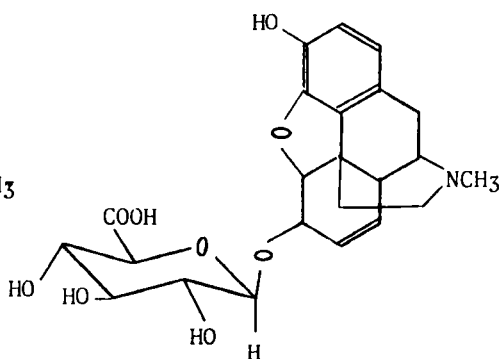


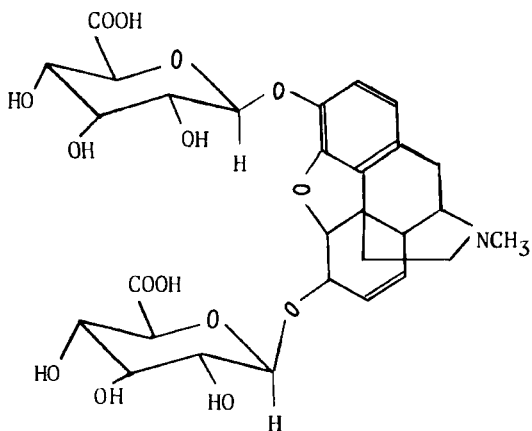
Fig.14 The chemical structure of prominent morphine metabolites



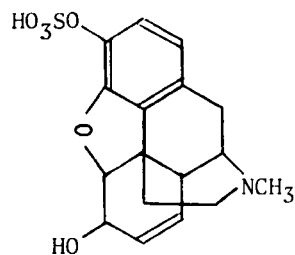
Morphine 3-glucuronide



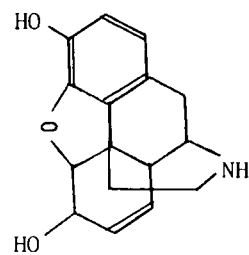
Morphine 6-glucuronide



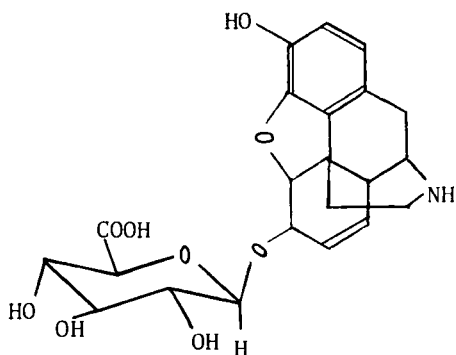
Morphine 3,6-diglucuronide



Morphine 3-ethereal sulfate



Normorphine



Normorphine 6-glucuronide

## 7. Pharmacokinetics

### 7.1 Plasma Levels

After parenteral administration of morphine (in doses of  $5.75 \text{ mg/m}^2$  of body surface area), the plasma levels of free morphine at 1 hour varied from 21  $\mu\text{g}$  per milliliter (for the intravenous) to 35  $\mu\text{g}$  per milliliter (for the intramuscular and subcutaneous injections). The levels then declined exponentially, reaching low levels at 6 and at 9 hours (83).

After oral administration of the same dose as above, the plasma levels of free morphine were significantly lower than after parenteral administration at all times except at 9 hours when they were similar (83).

At 2 hours the concentrations of conjugated morphine (resulted from the same doses as above) ranged from 148 to 164  $\mu\text{g}$  per milliliter, and at 24 hours, the levels ranged from 14 to 17  $\mu\text{g}$  per milliliter. The decline in plasma conjugated morphine is not exponential (83).

Following 0.2 mg/kg intrathecally and epidurally, plasma levels were 42 and 67 ng/ml respectively at 60 minutes (107).

Plasma morphine concentrations after epidural injection are similar to those found after intramuscular administration (108). Epidural administration of morphine resulted in cerebrospinal fluid (CSF) concentrations many times higher than those in plasma (108).

Buccal administration of morphine sulfate (13.3 mg tablets, equivalent to 10 mg morphine) produced peak morphine serum concentrations of 36 ng/ml at approximately one hour post-administration (109). In this study, intramuscular morphine (10 mg) produced peak levels of 44 ng/ml at one hour (109). However, plasma levels declined more slowly after buccal morphine tablets and remained above 10 ng/ml at 8 hours (109).

### 7.2 Half-Life

Morphine has a life-life of about 2 hours with a range of 1.9 to 2.6 hours (83,110-112).

A half-life of 2.9 was also reported (113).

It has been stated that, in young adults the half-life of morphine in plasma is about 2.5 to 3 hours, this value may be longer in older patients (82).

It has been also reported that age does not appear to effect half-life, but it may vary between individuals (112). Route of administration does not alter plasma half-life (83).

### 7.3 Peak Analgesia

Peak analgesia occur after morphine administration as follows:-

Within 60 minutes following oral administration.

20 to 60 minutes after rectal administration.

50 to 90 minutes following subcutaneous injection.

30 to 60 minutes after intramuscular injection.

Within 20 minutes after intravenous administration.

Analgesia may be maintained up to 7 hours (114).

### 8. Drug Stability

Morphine hydrochloride and morphine sulfate should be kept in a well-closed container, protected from light (11). Morphine hydrochloride in the dry state, stored as above, showed no decomposition after almost five years when examined by different physical and chemical methods including spectroscopic evidence (115).

Morphine sulfate, when exposed to air gradually loses its water of hydration. The drug darkens on prolonged exposure to light (114).

Morphine sulfate injection should be protected from light and stored at a temperature less than 40°C preferably between 15 to 30°C; freezing should be avoided (114).

Duramorph PF injection contains no preservatives and is intended for single use only, unused portion should be discarded.

Morphine sulfate soluble tablets, extended release tablets, and oral solution should be stored in tight, light-resistant containers at 15 to 30°C.

Morphine sulfate has been reported (114) to be physically or chemically incompatible with solutions containing aminophylline, amobarbital sodium, chlorothiazide sodium, phenytoin sodium, heparin sodium, meperidine hydrochloride, methicillin sodium, nitrofurantoin sodium, pentobarbital sodium, phenobarbital sodium, sodium bicarbonate, sodium iodide, sulfisoxazole diolamine, and thiopental sodium.

## 9. Methods of Analysis

### 9.1 Identification Tests

9.1.1 The following identification tests are mentioned under morphine hydrochloride in the British Pharmacopoeia (11).

- Add to 1 mg of the powdered substance in a porcelain dish 0.5 ml of sulfuric acid containing 0.05 ml of formaldehyde solution; a purple color is formed which turns to violet.

- Dissolve 5 mg in 5 ml of water and add 0.15 ml of a freshly prepared 1% w/v solution of potassium hexacyanoferrate and 0.05 ml of a 10.5% w/v solution of iron chloride hexahydrate; a bluish-green color develops immediately.

- Dissolve 5 mg in 5 ml of water, add 1 ml of hydrogen peroxide solution (10 vol), 1 ml of 6M ammonia and 0.05 ml of a 4% w/v solution of copper sulfate; a transient red color develops.

- Yields the reactions characteristic of alkaloids and the reactions characteristic of chloride mentioned in (11).

9.1.2 The following identification tests are mentioned under morphine sulfate in the United States Pharmacopoeia (17).

- The infrared absorption spectrum of a potassium bromide dispersion of it, previously dried at 145° for 1 hour, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Morphine Sulfate R.S.

- To 1 mg in a porcelain crucible or small dish add 0.5 ml of sulfuric acid containing in each ml, 1 drop of formaldehyde T.S.; an intense purple color is produced at once which quickly changes to deep blue-violet color.

- To a solution of 5 mg in 5 ml of sulfuric acid in a test tube add 1 drop of ferric chloride T.S., mix and heat in boiling water for 2 minutes; a blue color is produced, and when 1 drop of nitric acid is added, it changes to dark red-brown.

- A solution (1 in 50) responds to the tests for sulfate mentioned in USP XX.

9.1.3 The following color tests are used to identify morphine and salts.

- Nitric Acid Test:

Sprinkle a little of morphine on the surface of a drop of nitric acid; an orange red color is produced which changes immediately to yellowish orange.

- Nitrous Acid Test:

Dissolve few crystals of morphine in dilute hydrochloric acid, add few crystals of sodium nitrite, render alkaline with potassium hydroxide solution, a red to orange color is produced.

- Iodic Acid Test:

Dissolve a small amount of morphine in 0.1 ml sulfuric acid, add few drops of potassium iodate solution and 1 ml of chloroform and shake. The chloroform layer soon colored violet.

- Sucrose Test:

Mix 10 mg of morphine with 40 mg of finely powdered sucrose in a porcelain dish, add two drops of conc. sulfuric acid, a red color is developed which changes to violet.

- Donath's Test:

To about 10 mg of morphine in a porcelain dish, add few drops of sulfuric acid and a small particle of potassium arsenate, warm on a small flame; a blue violet color passing into dark brown-red will appear after further warming which changes into reddish color upon addition of water.

## 9.2 Microcrystal Formation

The microcrystals of morphine were performed on a solution of morphine sulfate in water (10 mg in 10 ml). One to two drops of this solution was treated with the specified reagent on a microscopical glass slide. After a specific time, the crystals so formed were microscopically examined. The microcrystal formations are presented in table 9 (116).

Table 9: Microcrystal Formation of Morphine

Plate	Reagent	Time of formation (after)	Shape of the Crystals
1	Sodium carbonate (5% aq.)	12 min.	Long radiating needles (also ref. 117)
2	Potassium iodide (5% aq.)	15 min.	Linear needles arranged in groups (also ref.118)
3	Potassium chromate (5% aq.)	14 min.	Rectangular plates single or radiating groups.
4	Sodium phosphate dibasic (5% aq.)	10 min.	Long prisms arranged in groups
5	Marm's (Potassium cadm. iodide)	Immediate	Thin radiating hairs (also ref. 26)
6	Calcium hydroxide (saturated aq.)	3 min.	Short rods of different sizes.
7	Mercuric chloride	2-3 min.	Feathery thin crystals
8	Mayer's	Immediate	Lobed crystals
9	Wagner's* (Potasium tri-iodide)	3-5 min.	Brown needles or plate arranged in rosettes (also ref. 26)

\*To perform this test 7 mg of morphine sulfate is dissolved in 1 ml water.

Shehata (116) has performed all above tests.



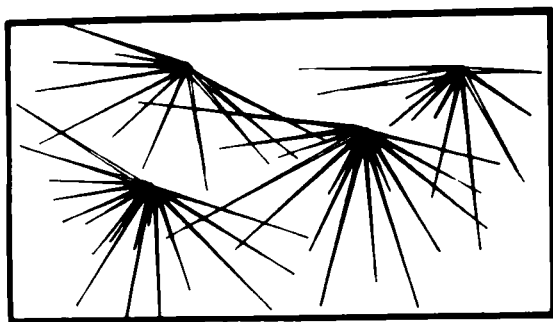


PLATE 1 : MICROCRYSTALS OF MORPHINE WITH SODIUM CARBONATE.

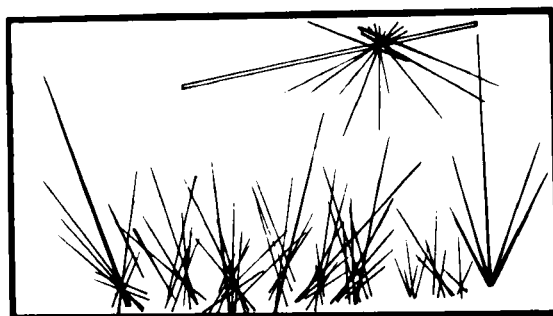


PLATE 2 : MICROCRYSTALS OF MORPHINE WITH POTASSIUM IODIDE.

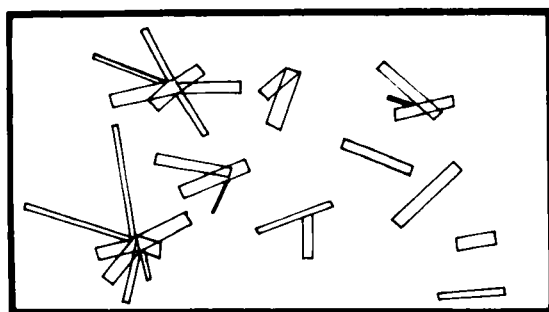


PLATE 3 : MICROCRYSTALS OF MORPHINE WITH POTASSIUM CHROMATE.

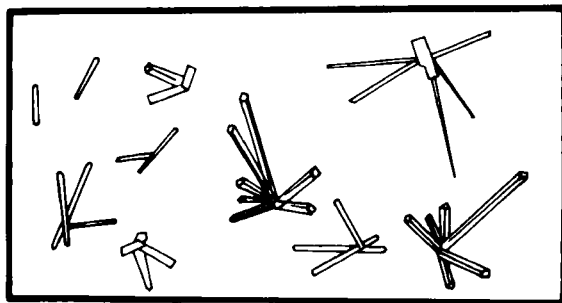


PLATE 4 : MICROCRYSTALS OF MORPHINE WITH SODIUM PHOSPHATE.



PLATE 5 : MICROCRYSTALS OF MORPHINE WITH MARM'S REAGENT.

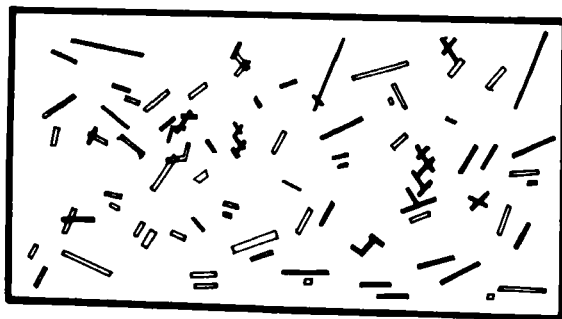


PLATE 6 : MICROCRYSTALS OF MORPHINE WITH CALCIUM HYDROXIDE.

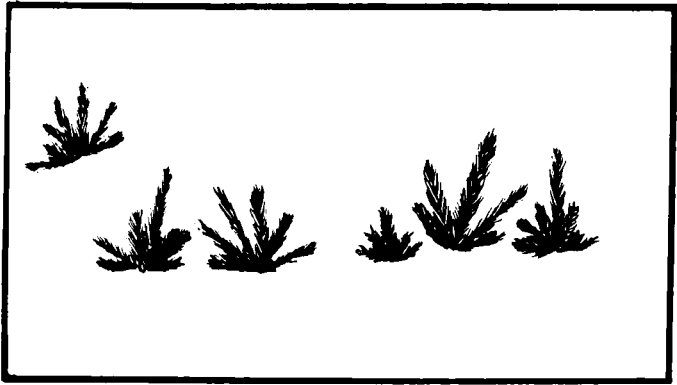


PLATE 7: MICROCRYSTALS OF MORPHINE WITH MERCURIC CHLORIDE.

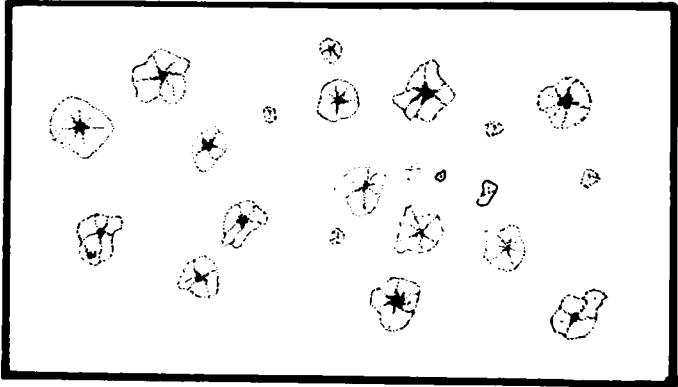


PLATE 8: MICROCRYSTALS OF MORPHINE WITH MAYER'S REAGENT.

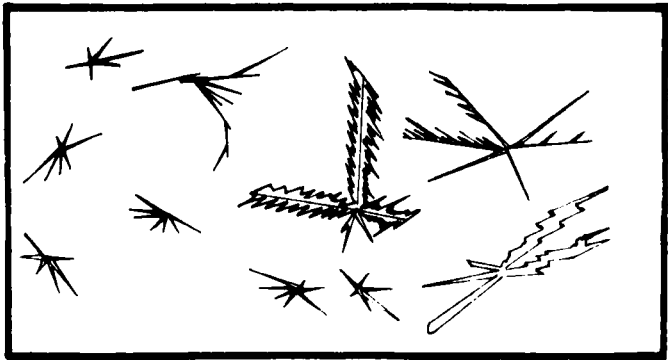


PLATE 9: MICROCRYSTALS OF MORPHINE WITH WAGNER'S REAGENT.

### 9.3 Titrimetric Methods

The official methods of assaying morphine salts and some of its formulations as well as morphine content in opium and in most of its preparations are titrimetric methods. Morphine formulations and most of opium preparations are assayed by the aqueous titration methods, whereas morphine salts are assayed by the non-aqueous titration techniques (11,17,119).

#### 9.3.1 Aqueous Titration Methods

Morphine sulfate tablets are assayed by the following method (119).

Accurately weigh and powder 20 tablets. Shake a quantity of the powder equivalent to 0.1g of morphine sulfate with 25 ml of water and 5 ml of N sodium hydroxide, add 1 g of ammonium sulfate, shake to dissolve, add 20 ml of alcohol (95%), and extract with 40,20,20 and 20 ml of a mixture of three volumes of chloroform and one volume of alcohol (95%). Wash each extract with the same 5 ml of water, filter and remove the solvent. Dissolve the residue in 10 ml of 0.05 N hydrochloric acid, boil, cool, add 15 ml of water, and titrate the excess of acid with 0.05 N sodium hydroxide using methyl red solution as indicator. *Each ml of 0.05 N hydrochloric acid is equivalent to 0.07897 g of morphine sulfate ( $C_{17}H_{19}NO_3$ )<sub>2</sub>,  $H_2SO_4$ ,  $5H_2O$ .*

Raw opium, powdered opium, opium extract and opium tincture are all assayed for their morphine content which is calculated as anhydrous morphine (11,17,119). The method involves extraction of the morphine from the sample with calcium hydroxide solution, washing with an ethanol-diethyl ether mixture, precipitation of the free base with ammonium chloride, dissolution of the base in a known volume of 0.1 sulfuric acid and titration of the excess of the acid with standard 0.1 N sodium hydroxide solution in the presence of methyl red as an indicator.

*Each ml of 0.1 N sulfuric acid is equivalent to 0.02853 g of anhydrous morphine.*

A correction is applied for the loss of morphine due to its solubility.

### 9.3.2 Non-Aqueous Titration

The B.P. (11) describes a non-aqueous titration method for the assay of morphine hydrochloride and morphine sulfate as follows:- Dissolve 0.4 g accurately weighed of the dried substance of morphine hydrochloride (0.5 g of morphine sulfate) in 30 ml of anhydrous glacial acetic acid, add 10 ml of mercuric acetate solution and drops of crystal violet solution as indicator.

Titrate with 0.1 M perchloric acid to the end point.

*Each ml of 0.1 M perchloric acid VS is equivalent to 0.03218g of  $C_{17}H_{19}NO_3 \cdot HCl$ .*

*or each ml of 0.1 M perchloric acid VS is equivalent to 0.06688 g of  $(C_{17}H_{19}NO_3)_2 \cdot H_2SO_4$ .*

The B.P. (11) requires the end point to be determined potentiometrically.

USP XX (17) describes the following method for the assay of morphine sulfate:-

*Sodium methoxide solution:-* Prepare as directed for 0.1 N sodium methoxide VS, but dilute with benzene to 2000 ml. standardize the solution daily.

*Sodium methoxide standardization:-* Dissolve about 250 mg accurately weighed of primary standard benzoic acid in 80 ml of dimethylformamide contained in a conical flask and observe precautions against absorption of atmospheric carbon dioxide. Add 3 drops of a 1 in 100 solution of thymol blue in dimethylformamide, and titrate with sodium methoxide solution to a blue end-point.

Each ml of 0.05 sodium methoxide VS is equivalent to 6.106 mg of benzoic acid.

*Procedure:-*

Weigh accurately about 700 mg of morphine sulfate, transfer to a conical flask, and dissolved in 80 ml of the same lot of dimethylformamide in the standardizing the sodium methoxide solution, observing precautions against absorption of atmospheric carbon dioxide. Add 1 drop of a 1 in 100 solution of phenol red in dimethylformamide and titrate with sodium methoxide solution to a red end-point.

*Each ml of 0.05 N sodium methoxide solution is equivalent to 16.72 mg of  $(C_{17}H_{19}NO_3)_2 \cdot H_2SO_4$ .*

An alternative method for determining morphine hydrochloride in non-aqueous medium was reported (120). Morphine hydrochloride (0.2 g) was dissolved in glacial acetic acid (5-10 ml), 3% solution of mercuric acetate (10 ml) was added. The resulting mixture was titrated with 0.1 N perchloric acid in the presence of genian violet indicator until bluish green end-point.

Another method for quantitative determination of morphine and morphine hydrochloride has been reported (121).

An accurately weighed sample is dissolved in 5 ml glacial acetic acid and titrated to the potentiometric end-point with 0.1 N acetous perchloric acid, using a glass indicating electrode and a calomel reference electrode filled with 0.02 N lithium chloride in glacial acetic acid. The titrant was standardized against dried potassium biphthalate. A blank titration is run. (Mercuric acetate is added to the titration medium in case of morphine hydrochloride to convert all chloride salts to titratable bases).

A non-aqueous titration procedure for the determination of narcotics including morphine was described (122). An accurate quantity of morphine is dissolved in a mixture of chloroform (20 ml) and phenol (2 ml) and titrated with 0.005 N toluene-p-sulfonic acid in a mixture of chloroform-dioxane, using methyl orange as an indicator.

A procedure for the determination of morphine in non-aqueous media was also described (123). It depends on solvent extraction of morphine followed by partial purification, and upon treatment with *1-chloro-2,4-dinitrobenzene* to form the dinitrophenyl ether derivative of morphine. This is titrated in phenol-chloroform solution with *toluene-p-sulfonic acid* in chloroform, using dimethylamino-benzene as indicator.

#### 9.4 Gravimetric Determination

Several methods have been reported for the determination of morphine gravimetrically. One of these methods proposed by Mannich in 1935 (124) and depends on the formation of the *dinitrophenyl ether derivative of morphine* upon the reaction of morphine with *1-chloro-(or 1-fluoro)-2,4-dinitrobenzene* in the presence of a base. Many modifications of this technique were elaborated (125-133), and even this method was introduced to some pharmacopeias (134). In summary this method can be performed as follows:- Morphine is extracted from opium or from any of its galenical preparations and then is subjected to purification by elution through an alumina chromatographic column. The eluate which contains morphine is usually treated with *1-fluoro-2,4-dinitrobenzene* in acetone for 4 hours at room temperature. The morphine dinitrophenyl ether so formed, is filtered, washed with acetone, dried and weighed. Schultz and Schneckenburger (130) has reviewed the modifications of this technique. It was found that loss of morphine may result from the slight solubility of the dinitrophenyl ether (130,135). Improved techniques were also reported to overcome this loss (130,131,133).

The morphine dinitrophenyl ether formed as above can be determined by titrimetric methods, either using acid-base titration (136) or by the non-aqueous titration technique (123).

- Certain organic bases including morphine can be precipitated with a solution of molybdophosphoric acid (137). The precipitate is then collected, dried and weighed.

## 9.5 Electrochemical Methods

### 9.5.1 Polarographic Determinations

Several polarographic methods for the determination of morphine have been reported (138-147).

In one method, the determination depends on treating morphine hydrochloride with nitrile to form the *2-nitroso morphine* which is readily determined polarographically, the amount of morphine is determined from a calibration graph prepared from known amounts of morphine standard (138-142,147). This method has been modified to determine small amounts of morphine in blood and plasma as follows (140): The sample is deproteinized with uranyl chloride and the supernatant fluid after centrifugation is acidified and autoclaved at 120° for 30 minutes. The resulting solution is saturated with NaHCO<sub>3</sub> and extracted with CHCl<sub>3</sub>-isopropanol (3:1). The solvent extracts are distilled to dryness and redissolved in N HCl and is treated with nitrile for polarographic determination of the 2-nitrosomorphine.

Other methods include:-

- Oscillopolarographic behavior of morphine as well as papaverine, apomorphine and cocaine (143).
  - Anodic determination of morphine by oxidation at rotating platinum electrode (144).
  - Polarographic determination of morphine and its derivatives by using a supporting electrolyte of 0.4 N H<sub>2</sub>SO<sub>4</sub> (145).
- Determination of morphine using a self-cleaning rotatory silver electrode (145).

### 9.5.2 Potentiometric Determination

Morphine and its official salts (the hydrochloride and the sulfate) are titrated to the potentiometric end point (11,17,121 ).

### 9.5.3 Conductimetric Determinations

A method was reported for the determination of morphine hydrochloride conductimetrically (148).



0.1% solution of morphine HCl (2 ml) is mixed with 10% aqueous ammonia (1 ml) and extracted with chloroform-ethanol (3:1) (3x10 ml, then 2x5 ml).

The combined extracts are evaporated, dissolved in a mixture of ethanol (20 ml), water (5 ml) and titrated conductimetrically (platinum electrodes) with 0.01 N HCl and end point is determined graphically.

The hydrochloride of morphine, cocaine and pilocarpine were determined by means of a chloride-selective electrode as well as potentiometric titrimetry for comparison (149):- In the first method, portions of 10 mM solution of each separate drug were diluted to 100 ml with water mixed with 2 ml of 5M AgNO<sub>3</sub> and stirred for 3 minutes. These were determined by means of a *Cl<sup>-</sup>-selective indicator electrode*.

The measured values were used to prepare calibration graphs.

In the second technique, each sample was diluted with water and potentiometrically titrated with 0.1M AgNO<sub>3</sub>, the end points were determined graphically.

Results were obtained for each drug indicate good precision and accuracy of both techniques but the potentiometric titrimetry was preferred.

## 9.6 Spectrophotometric Methods

### 9.6.1 Colorimetry

A colorimetric method based on the color of the nitrosocompound formed by the reaction of nitrous acid with morphine was reported (150).

This method is still official in the B.P. (11) and used to assay morphine content in camphorated opium tincture. Originally the method was described as follows:-

Two samples (*each containing 2 mg of morphine*) were pipetted into volumetric flasks (each 25 ml) and the total volume of each was brought up (approximately 5 ml). To each sulfuric acid (3 ml of 1 volume concentrated  $H_2SO_4$  and 4 volumes of water) was added. 1% sodium nitrite solution (2 ml) was added to one flask (the other was served as a blank).

After the flask stood for 10 minutes at room temperature, *N NaOH solution* (4 ml) was added to each flask, allowing the flasks to cool in an ice water bath (for 5 minutes). The cooled solutions were diluted to volumes and the color was read in a suitable colorimeter.

- Modification of this method is described in the B.P. (11) as follows:-

The residue of morphine (*extracted from a known volume of the tincture*) is dissolved in *M hydrochloric acid* (10 ml), filtered, and sufficient water is added (to produce 50 ml). A volume of this solution (10 ml) is further diluted with water (to 20 ml) and a *freshly prepared 1% w/v solution of sodium nitrite* is added (8 ml). The resulting mixture is allowed to stand in the dark (for 15 minutes). *Ammonia solution 5 M* is added (12 ml) and this is diluted with water (to 50 ml). The absorbance of the resulting solution (in a 4-cm layer cell) is measured at 442 nm.

A blank is prepared in a similar manner, but using water (8 ml) instead of the solution of sodium nitrite.

The concentration of morphine is then calculated by reference to a calibration curve prepared by using 2-, 4-, 6-, and 8-ml quantities of a 0.008% w/v solution of anhydrous morphine in 0.1M hydrochloric acid and treated similarly.

- A colorimetric routine method was described for the determination of morphine based on the *iodic acid-ammonium carbonate-nickel salt reaction* (151) as follows:-

A solution of morphine (15 to 30 mg per 100 ml) is adjusted to pH 4 to 5. An aliquote (10 ml) is treated for exactly 2 minutes with 4.5% w/v iodic acid solution (2 ml); then with the complex reagent (10 ml) [The reagent consists of 8.0 g ammonium carbonate shaken with 25 ml of 21.4% w/v  $\text{NH}_4\text{Cl}$  solution, 20 ml of 0.1 N aqueous ammonia and 10 ml of 1.0% w/v  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  solution diluted to 100 ml and prepared freshly each day].

The resulting solution is diluted to 25 ml and the extinctions at 530 and 670  $\mu\text{m}$  are determined after 90 minutes. (The pH of the end of this period should be  $8.0 \pm 0.05$ ).

A blank determination is carried out with 0.1 N HCl (5 ml) in place of iodic acid solution.

The morphine content is determined by reference to a calibration curve at 670 nm prepared from solutions containing 4.0 to 40 mg of anhydrous morphine per 100 ml.

- Another colorimetric method was developed to estimate morphine content in Indian opium (152). This method depends on the *pink copper complex of morphine* early described by Oliver (153) as a color test for morphine. The procedure is as follows:- An accurate quantity of morphine solution (1 ml) is diluted with acidified methanol (to 5 ml). To this solution, a mixture consists of hydrogen peroxide solution (2 ml), cupric chloride solution (2 ml) and 15 M ammonia (1 ml) is added. The resulting mixture is thoroughly shaken and after 30 minutes, the absorbance of this mixture is measured in a suitable spectrophotometer against a blank at 475 nm.
- An old procedure for the determination of morphine in syrup was described (154):- To morphine syrup (10 ml), 10%  $\text{HIO}_3$  (1 ml) was added, this was shaken and the color was allowed to develop for 5 minutes, concentrated ammonia solution (1 ml) was added and the mixture was again shaken and allowed to stand for 45 minutes. The intensity of the final red brown color was compared with that obtained in a parallel experiment with a syrup prepared according to the French Codex.

- A rapid colorimetric method for the estimation of morphine from biological materials has been reported (155). Morphine is extracted with n-butanol from the materials made strongly alkaline with potassium hydroxide solution. The final extract is treated with molybdo-silicic acid followed by aqueous ammonia and the resulting color is measured at 675 nm.
- Morphine in minute quantities (3  $\mu$ g of the free base in 1g of blood or tissues or 0.6  $\mu$ g per ml of plasma) can be determined colorimetrically as follows (156). After removal of impurities with benzene followed by precipitation of morphine and adsorption on ion exchange resin. It can be determined colorimetrically by Folin Ciocalteu phenol reagent.

This method has also been used in combination with thin layer chromatography (TLC) as follows (157):-

Known amounts of opium extract were applied as spots on TLC plates, after development of the chromatoplates in the solvent system, the morphine spots were scrapped off, extracted with 0.1 N HCl and to this extract, *Folin-Ciocalteu reagent* was added. The extinction of the resulting color was measured at 660 nm and morphine was calculated from a calibration graph.

- Morphine sulfate can be colorimetrically determined by the following procedure:- (158 )  
1% methanolic solution of diazotized-4-amino-6-chlorobenzene-m-disulphon-amide reagent (1 ml) is mixed with both 1% Na NO<sub>2</sub> solution (1 ml) and N-HCl (1 ml) and kept aside for 2 minutes.  
Aqueous solution of morphine sulfate (1 ml containing < 1 mg) and N NaOH are added to the previous mixture, shaken and kept aside for 5 minutes. The resulting mixture is then diluted to 10 ml with water and the extinction of this mixture is measured at 510 nm.

(Sucrose, lactose, papaverine, codeine and dionin do not interfere with the assay).

- Other colorimetric methods for the estimation of morphine were also reported (159-167).

Estimation of morphine in biological materials was described by several authors (155,156,165-167).

In one procedure it involves the esterification of morphine with p-nitrobenzoylchloride and extracting the ester into ethylene dichloride the ester is determined by complexing with methyl orange or with bromocresol purple (166 ).

9.6.2 Ultraviolet (UV)

A UV absorption method for the determination of morphine in opium has been reported (168,169).

Elution of opium alkaloids was effected through three chromatographic Celite columns, in 0.1 M citric acid-sodium citrate buffer at pH 4.4, in M  $K_2HPO_4$  and in 0.5 M NaOH respectively.

The morphine content was calculated from the extinction at the maximum at 285 nm, and corrected for the base line by extrapolation of the straight part of the curve between 310 and 340 nm to a point below the maximum.

- A similar UV method for the determination of morphine in paregoric was described (170). The morphine is extracted from 5 ml sample into chloroform-ethanol mixture.

The absorption spectrum of the resulting solution of morphine is recorded between 250 and 350 nm in both acid and alkaline aqueous solutions.

The extinction at the maximum of the alkaline spectrum is used and corrected for base line by extrapolation of the straight part of the curve between 330 and 350 nm to a point below the maximum.

- Morphine sulfate can be determined in the presence of atropine sulfate by measuring the UV absorption at 285 nm;  $A(1\%, 1\text{ cm})=40$  (171).
- Morphine, papaverine and ephedrine alkaloids, each can be determined with the aid of short-wave UV radiation (172).

The absorption of each alkaloid is measured at 253.7 nm which obtained from low pressure mercury lamp in conjunction with luminescent substance at pH 1.9. The molecular extinction coefficient,  $c$  for morphine is  $9.486 \times 10^2$ , for papaverine is  $5.767 \times 10^4$  and for ephedrine is  $1.353 \times 10^2$ .

- Another procedure for the assay of morphine in opium has been reported (173). Morphine is eluted through acid and basic chromatographic columns respectively. Morphine eluent is collected and measured at 303 nm.
- A method for the determination of morphine suppositories by its UV absorption has been reported (174). Morphine suppository (containing 10 mg morphine) in light petroleum (25 ml) is extracted with water (5x15 ml each containing one drop of 2 M HCl). The aqueous phase is filtered off, made up to 100 ml and the extinction of the resulting solution is measured at 284.5 nm.

Morphine content is calculated from a calibration curve (which can be done up to 20 mg morphine per 100 ml).

Morphine formulations (*tablets, suppositories and ear-drops*) have been assayed for morphine content using the  $\Delta A$  method to eliminate interferences (175). The absorbance at 298 nm is measured for solutions in acid and alkaline media, the difference ( $\Delta A$ ) being taken as a measure of morphine content (as it is proportional to morphine concentration). This method has been applied to suppositories after extraction of the drug from the base. Ear drops containing phenol in addition to morphine and these are assayed by measuring  $\Delta 3$  at 288 and 298 nm and evaluating the morphine concentration from a pair of simultaneous equations (175).

*Assay for tablets.* An accurately weighed quantity of the powder (representing 10-20 tablets) was quantitatively extracted with 0.1N sulphuric acid, filtered into a 100 ml volumetric flask and subsequently made to volume. Two 10 ml portions of the filtrate were diluted to 100 ml using 0.1N sulphuric acid and 0.1N sodium hydroxide respectively. The absorbances of 1 cm pathlengths of both solutions were measured at 298 nm.

- Other UV absorption methods have been reported (176-179).

### 9.6.3 Spectrofluorimetry

A spectrofluorimetric method is described for the detection and determination of morphine, codeine, narcotine (noscipine) and papaverine in mixtures (180).

Morphine and codeine can be determined in aqueous media by differential method as follows:-

The alkaloid mixture is accurately weighed (40 to 50 mg) and dissolved in 0.1 N sulfuric acid (100 ml). An aliquot (10 ml) is transferred into a 50 ml standard flask and diluted to volume with 0.1 N sulfuric acid (solution A). Another aliquot (10 ml) is transferred into second 100 ml standard flask and diluted to volume with 0.1 N sodium hydroxide (solution B).

Each solution (A and B) is treated with oxygen-free nitrogen for 10 minutes. The fluorescence intensities of both solutions are then measured at 285/345 nm. The concentrations of morphine and codeine are calculated from calibration graphs.

Another rapid and sensitive fluorimetric method for the estimation of submicrogram quantities of morphine (as little as 0.1 g) in biological tissues has been described (181). The basis of this method is the conversion of morphine, which is weakly fluorescent, to pseudomorphine which is highly fluorescent.

The method involves the extraction of morphine into a chloroform-butanol mixture from tissues saturated with sodium bicarbonate, and the re-extraction of morphine into acid solution. Morphine is then oxidized to pseudomorphine by the use of potassium ferricyanide in a weakly alkaline solution. The fluorescence so produced is measured in a 1-cm quartz cavette at 440 nm by using a suitable spectrofluorimeter.

Several other spectrofluorimetric methods have been reported (182-186).

#### 9.6.4 Infrared Spectroscopy (IR)

Morphine and codeine in opium can be rapidly and accurately determined simultaneously by IR in carbon tetrachloride after quantitative instantaneous acetylation (187):-

Accurately known amounts of opium are extracted. Morphine and codeine are separated and purified by elution through alumina chromatographic column. Liquid-liquid extraction of the alkaloids is effected and these are acetylated, dissolved in carbon tetrachloride and their IR spectra are measured. The percentage of morphine (M) and codeine (C) are calculated according to the following equations:-

$$M \% = \frac{A_2}{0.0306} \times f$$

$$C \% = (24.2 A_1 - 21.6 A_2) \times f$$

$$\text{where } A_2 = A_{1038} - A_{1025}$$

$$A_1 = A_{1058} - A_{1087}$$

$$f = 1.11$$

$$A = \text{Absorbance}$$

#### 9.6.5 Mass Spectroscopy

Opium alkaloids can be determined from their mass spectra by comparison of the parent peak of each individual alkaloid with that of a reference substance added in known amount and by reference to a calibration graph (188,189). At a low ionization voltage, the  $M^+$  ion can be detected clearly, but the peak is small. At higher ionization voltages, the  $M^+$  ion peak becomes higher, but many other fragment peaks overlap it.

The relationship between the intensity of the  $M^+$  ion peak and ionization voltage was studied and appropriate ionization voltages were determined.

#### 9.6.6 Gas Chromatography/Mass Spectrometry (GC/MS)

Data identifying the major alkaloids of opium were obtained by computer monitored GC/MS. This technique is applicable for forensic identification of the major opium alkaloids particularly morphine (190).

Another study includes the identification of morphine and codeine in opium by using GC/MS technique. In this study a sample of opium containing 2 mg morphine and 0.5 mg codeine can be easily analyzed (191).

Morphine, codeine and hydromorphone can be quantitatively determined in blood or serum by GC/MS (192).

Morphine, codeine and hydromorphone were extracted from blood or serum using a one step extraction. The extraction was derivatized and the trifluoroacetyl opiates were quantified by GC/MS, selected ion monitoring using nalorphine as the internal standard was applied. Calibration curves were obtained and these were linear.

Sensitivity as low as 0.02 mg/l for morphine and codeine and 0.08 mg/l hydromorphone was achieved. Urine or tissue homogenates could be processed similarly after acid hydrolysis (192).

A procedure for the assay of morphine codeine and other potential urinary metabolites by GC/MS has been also reported (193).

Other GC/MS method was also reported (194).



## 9.7 Chromatography

### 9.7.1 Paper Chromatography

Several paper chromatograms have been used for the identification and quantitation of morphine. Some of these are listed in table 10.

Table 10: Paper Chromatography of Morphine

Chromatogram	Solvent	Rf value	Reference
Whatman No.1, buffered by dipping in 5% solution of sodium dihydrogen phosphate, blotting and drying at 25° for 1 hour.	4.8 g of citric acid in a mixture of 130 ml water and 870 ml n-butanol.	0.14	(26)
Reversed phase, Whatman No.1, impregnated with 10% solution of tributyrin in acetone and drying in air.	Acetate buffer (pH 4.58) or Phosphate buffer (pH 7.4)	0.88 0.88	(26,195) (26,196)
Whatman No.1, impregnated with 0.5 M $\text{KH}_2\text{PO}_4$ (pH 4.2)	Cyclohexane-chloroform-diethylamine (7:2:1)	0.55	(197)
Paper impregnated with 0.1 M citric acid and with 0.2 M $\text{Na}_2\text{HPO}_4$ buffer, (pH 3.4-3.9).	Isobutanol-toluene-ethanol-water	-	(198)

The spots in the above chromatograms are visualized with one of the followings (26):

- Examination under UV (254 nm).
- Spraying with Iodoplatinate reagent.
- Spraying with Dragendorff's reagent.
- Exposure to iodine vapor.

### Quantitative Paper Chromatography

Morphine can be determined quantitatively by the use of paper chromatography (197,199-202).  
A descending technique on Whatman No.1 paper strips (197).

The paper is impregnated with a solution of ammonium sulfate (2%). Freshly prepared solvent system consists of *isobutanol-acetic acid-water* (10:1:2.4) is used. The aqueous solution of morphine hydrochloride (or ethanol solution of morphine) is spotted by means of an "Alga" micrometer syringes. Five microliteres, which should contain 5-50  $\mu\text{g}$  of the alkaloid is applied, yielding a spot not larger than 5 mm in diameter. The paper is equilibrated for 6 hours in a jar which is saturated with the solvent vapors.

Chromatography takes place over-night (16 hours), in which time, the solvent front travels about 38 cm. After drying, the chromatogram is sprayed on both sides thoroughly and uniformly with potassium iodoplatinate reagent and dried again for 15 minutes in a current of air.

The total color density of the blue spots on the white background are scanned directly by utilizing a self-integrating densitometer.

The standard curve of morphine hydrochloride (or of morphine) is prepared by plotting the concentrations (in  $\mu\text{g}$ ) vs total density of the spots (197).

#### 9.7.2 Thin Layer Chromatography (TLC)

TLC are used for fast and reliable identification of morphine and its metabolites. Many chromatograms have been reported and several of these are presented in table 11.

Table 11: Thin Layer Chromatography of Morphine

Chromatogram	Solvent System	Rf	Reference
1. Silica gel G (0.25 mm layers)	Methanol-strong ammonia solution (100:1.5)	0.34	(26,203)
2. Silica gel G	Benzene-dioxane-ethylalcohol-strong ammonia solution (50:40:5:5)	0.11	(26,176)
3. Silica gel G	Acetic acid (glacial)-ethylalcohol-water (30:60:10)	0.27	(26,176)
4. Silica gel G	Xylene-ethylmethylketone-methanol-diethylamine (20:20:3:1)	0.10	(204)

Chromatogram	Solvent System	Rf	Reference
5. Silica gel G	Xylene-acetone-ethanol-ammonia (0.88) (20:20:3:1)	0.12	(205)
6. Silica gel G	Toluene-acetone-ethanol-ammonia (0.88) (20:20:3:1)	0.19	(205)
7. Silica gel HF <sub>254</sub>	Toluene-acetone-95% ethanol-25% aqueous ammonia (40:40:6:2)	-	(206)
8. Silica gel HF <sub>254</sub>	Toluene-acetone-ethanol, 25% ammonia solution (45:45:7:3)	0.1- 0.15	(206)
9. Silica gel G	Chloroform-methanol (33:66)		(207)
10. Silica gel G	Ethanol-dioxane-benzene- ammonium hydroxide (5:40:50:5)	-	(208)
11. Silica gel G	Chloroform-dioxane- ethylacetate/ammonium hydroxide (25:60:10:5)	-	(208)
12. Alumina GF	The lower phase of Chloroform-methanol- water-acetic acid (20:10:20:2)	0.40	(104)
13. Alumina GF	The lower phase of Chloroform-ethanol-water- acetic acid (20:10:15:1)	0.80	(104)
14. Alumina GF	The lower phase of Ethylene dichloride- propanol-water-acetic acid (20:10:10:1)	0.90	(104)
15. Silica gel F <sub>254</sub>	Acetone-toluene-ethanol -25% ammonia (40:40:12:2.5)	0.25	(79)
16. Cellulose F <sub>254</sub>	n-Butanol-acetic acid-water (35:3:10)	-	(79)
17. Cellulose F <sub>254</sub>	Propanol-ethylacetate- water (4:3:2)	-	(79)
18. Gelman's S-G* instant thin layer chroma- tographic paper	n-Butanol-water-acetic acid (35:3:10)	0.77	(83)

\*This system was also employed for the identification of some morphine metabolites.

Rf values: morphine 3-glucuronide 0.17; morphine ethereal sulfate 0.30.

The spots in the above chromatograms are visualized by one of the followings (26):

Examination under UV 254 light

Spraying either with potassium iodoplatinate reagent or Dragendorff's reagent.

Exposure to iodine vapors.

- Two-dimensional TLC technique was employed to identify morphine and some of its metabolites (normorphine, codeine and norcodeine). The solvent ethanol-benzene-dioxane-concentrated aqueous ammonia (50:40:5:5) was used as the first solvent system and the solvent dioxane-chloroform-ethylacetate-concentrated aqueous ammonia as the second mobile phase. The solvent front was 10 cm. in both direction.

Rf values: morphine 0.44 and 0.20

normorphine 0.19 and 0.05

codeine 0.56 and 0.41

norcodeine 0.27 and 0.19 (102).

- Quantitative TLC for the determination of morphine (209) and codeine (210) were reported. The same technique used in paper chromatography can be applied on TLC.

Other TLC systems for morphine were also reported (121, 176, 208).

### 9.7.3 Electrophoresis

This technique was first applied for the separation of aminoacids by high tension paper electrophoretic method (211). The same technique was applied for the detection of drugs of addiction in urine i.e. *morphine, cocaine, codeine, ethyl-morphine and diamorphine* (212) the technique used for these drugs is as follows:-

Sheets of filter paper which contain urine samples were suspended in hexane and the ends dipped into electrode vessels containing 10% solution of acetic acid which used as buffer. Platinum electrode were used and 1500 to 6000 volts were applied for 90 minutes. Separation of spots were effected.

Another method was reported for the separation and detection of opium alkaloids (213).

Paper chromatograms were impregnated in a buffer solution and developed in the solvent system butanol saturated with the buffer. 500V, 0.5 to 2mA per cm. in 1% aqueous ammonia were applied.

## 9.7.4 Gas Liquid Chromatography (GLC)

GLC affords one of the most reliable methods of identification of pure substances, their metabolites or a mixture with several components. GLC have been widely described for the identification of morphine and its metabolites in various biological fluids as well as the identification and quantitation of morphine and the other alkaloids of opium. Some of these systems are presented in table 12.

Table 12: The GLC of Morphine

Column Condition	Carrier Gas	Detector	Retention time ( <i>t<sub>R</sub></i> )	Reference
1. 1% SE-30 on 100-120 mesh Anakrom ABS (6 ft. x 4 mm internal diameter) Column temperature 250°.	Argon gas flow 80 ml/min.	AID or FID Hydrogen flow 50 ml/min.; air flow 300/min. (FID)	1.28 (relative to codeine)	(26, 214)
2. 2.5% SE-30 on 80-100 mesh Chromosorb W (5 ft. x 4 mm internal diameter) Column temperature 225°.	Nitrogen gas flow 50 ml/min.	FID, Hydrogen flow 50 ml/min; air flow 300 ml/min.	1.15 (relative to codeine)	(26, 215)
3. 5% SE-30 on 60-80 mesh Chromosorb W AW (5 ft. x 1/8 inch internal diameter) Column temperature 230°.	Nitrogen gas flow 30.7 ml/min.	FID, Hydrogen flow 22 ml/min.	1.21 (relative to codeine)	(26, 216)
4. Chromosorb G, (100-120 mesh acid washed and silanized), coated with a mixture of 0.35% JXR and 0.35% CDMS (column 0.9 m long and 4 mm internal diameter). Column temperature 210°.	Nitrogen hydrogen/air 60:60: 800 ml/min.	FID	Relative to nalorphine	(217)

Column Condition	Carrier Gas	Detector	Retention time ( <i>t<sub>R</sub></i> )	Reference
5. Methylsilicon gum on silanized acid wash flux-calcinated diatomite Column temperature 150-250°.	Helium at 50 ml/min.	FID	-	(121)
6. Two glass U-tube columns packed with 3% OV-17 on 100-120 Gas-Chrom Q and with 3.8% silicon Rubber UC-W98 on Chromosorb W.H.P. 80-100 mesh Injection port temperature at 280°.	Helium at 40 ml/min.	FID (maintained at 280°)	0.69 (in reference to nalorphine)	(218)
7. Dual columns Glass column (0.9 m x 2 mm) packed with 3% OV-17 coated on 60-80 mesh gas chrom Q. Stainless steel column (1.5 m x 2 mm) packed with 3% SE-30 coated on 100-120 mesh Varaport. Column temperature 220°, injector temperature 225°.	Nitrogen 30 ml/min; Hydrogen 30 ml/min; dried compressed air 300-400 ml/min.	Dual FID	6.0	(94,98)
8. Column packed with a 50:50 mixture of OV-17 phenylmethyl silicone (50% phenyl) on 80-100 mesh Varaport 30. (from Varian)	Helium 30 ml/min.; hydrogen pressure, 10 p.s.i.g.; air pressure, 40 p.s.i.g.	HFI (hydrogen flame ionization) (temperature 270°)	4.25 (relative to didecyl phthalate)	(219)

Column Condition	Carrier Gas	Detector	Retention time ( $t_R$ )	Reference
SE-30 silicone gum rubber on Chromosorb W(AW,DMCS) (from Perkin Elmer). Injector temperature 310°; oven temperature 250°-280°.				
9. Coiled glass column (1.83 m x 4 mm) packed with 3% OV-17 on Gas-chrom Q (100-120 mesh) Column temperature 200-220°, Injector temperature 275°.	Nitrogen 100-120 ml/min.	$^{63}\text{Ni}$ electron capture (ECD) temperature 300°	Morphine (H FB) <sub>2</sub> 4.1 (Relative to chlorpromazine)	(220)
10. Glass U-tube packed with Gas-Chrom P, 60-80 mesh, (washed with concentrated HCl, methanolic potassium hydroxide, dried and treated with hexamethyldisilazane. The dried and siliconized support was coated with 0.1% polyethylene glycol 9000 and finally with 4% silicone rubber SE-30. Column temperature at 183°.	Gas flow at 250 ml/min.	Argon ionization detector (AID)	-	(221)
11. Chromosorb G-HP AW-DMCS, 80-100 mesh (1.5 m x 2.3 mm internal diameter) Column temperature 150-235°.	Hydrogen 50 ml/min; air 290 ml/min.	FID	4.82 (Relative to antipyrine)	(222)

Column Condition	Carrier Gas	Detector	Retention time ( <i>t<sub>R</sub></i> )	Reference
12. 1:1 Mixture of 3% OV-17 on 80-100 mesh Varaport and 5% SE-30 on 80-100 mesh Chromosorb W (AW DMCS) (6 ft x 2 mm I.D.) Column temperature 190 to 240°	Helium 30 ml/min, hydrogen 44 ml/min, air 300 ml/min	FID at 300°	Morphine, Heroin and Cocaine relative to didecyl phthalate	(223)

Several GLC methods have been employed to determine morphine and morphine metabolites in biological fluids and tissues. Some of these methods are listed in the following table 13.

Table 13: GLC of Morphine in Biological Fluids and Tissues.

Recommended For	Conditions of Analysis	Reference
1. Estimation of morphine in 0.1 to 1.0 ml of plasma or cerebrospinal fluid.	Derivatization of the sample extract residue with trimethylsilyl-ether (TMS).	(224)
2. Quantitative determination of morphine in whole blood.	Derivatization of morphine with TMS.	(225)
3. Determination of morphine and codeine ( <i>both as free and conjugates</i> ) in post-mortem specimens ( <i>liver, bile and urine</i> ).	TMS derivatization of morphine and codeine	(218)
4. Characterization of morphine metabolites in humans	As trimethylsilyl derivatives	(94)



Recommended For	Conditions of Analysis	Reference
5. Quantitative determination of morphine in urine.	Forming the silylethers and employing nalorphine hydrobromide as an internal standard.	(217)
6. Determination of submicrogram amounts of drugs (including morphine) in biological fluids ( <i>Analysis of 2.5 ml urine sample containing 7.5 µg morphine and nalorphine per ml</i> ).	Direct application of morphine	(226)
7. Isolation and identification of morphine from post-mortem specimens ( <i>urine, bile, blood, liver, kidney, brain, spleen, spinal, ocular and amniotic fluids</i> ).		(227)
8. Quantitative determination of morphine at therapeutic levels in 1 to 2 ml of serum of plasma. Morphine levels less than 25 ng/ml can be effectively measured. ( <i>The procedure can be utilized in pharmacokinetic studies of morphine</i> ).	Morphine and nalorphine (as internal standard) both are measured by electron capture detection ( $^{63}\text{Ni}$ ) as their trifluoroacetyl derivatives.	(228)
9. Quantitative determination of morphine in plasma and brains samples down to 500 pg ml <sup>-1</sup> in plasma and 100 pg ml <sup>-1</sup> in 30 mg of brain tissues.	Derivatization of morphine to pentafluoropropionic derivative (PFP-morphine) and using electron capture detector.	(229)

Many other GLC systems were also reported for morphine (230-248).

### 9.7.5 High Pressure Liquid Chromatography (HPLC)

System 1      The following system has been employed for the detection, separation and quantitative determination of morphine, heroin and other narcotic analgesic drugs (249).

Column:          Zipax SCX (strong cation exchanger),  
2 mm ID x 80 to 100 cm in length.

Mobile Phase:   NaOH-H<sub>3</sub>BO<sub>3</sub> buffer with pH values from  
9.2 to 10.0 and ionic strengths from  
0.02 to 0.2 M.

Detection:      Mass spectrometry or UV spectroscopy  
(for morphine at 230 nm).

System 2      The following system has been reported for the purity profile of morphine, morphine hydrochloride and heroin in order to define the scope of utility of each as qualitative and quantitative analytical standards (121).

Column:          SCX (chemically bonded sulfonated  
fluorocarbon polymer), 1.0 m x 2.2 mm  
ID; temperature 35°C.

Mobile Phase:   Gradient elution 0.4-1.4 M sodium  
perchlorate in 0.01 M pH 6.8 phosphate  
buffer containing 10% ethanol.

Detection:      UV absorption at 254 nm.

System 3      This system is employed for the routine analysis of the five major alkaloids of gum opium and for the quantitation of morphine, codeine and thebaine by isocratic reverse phase HPLC. (250).

Column:           $\mu$ -Bondapak C<sub>18</sub>/Porasil, 30 cm x 4 mm  
ID.

Mobile Phase:   0.1 M NaH<sub>2</sub>PO<sub>4</sub> in 5% CH<sub>3</sub>CN/H<sub>2</sub>O.

Flow Rate:      1.25 ml/min.

Detection:      254 nm UV.

System 4

This system has been also employed for the identification and quantitative determination of the five major alkaloids of opium (251).

Column: 5  $\mu$ m Porasil, 30 cm x 4 mm ID, oven temperature maintain at 25°C.

Mobile Phase: Methylene chloride-ethanol-diethylamine (30:40:0.5).

Flow Rate: 2.4 ml/min.

Detection: UV at 285 nm.

System 5

The following reversed phase system has been used for the analyses of morphine and its metabolites as well as heroin and its metabolites (252).

Column:  $\mu$ -Bondapak C<sub>18</sub> Porasil.

Mobile Phase: 55:45 methanol-buffer, with the pH 6.98 buffer containing 0.1% (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> and 0.01 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>.

Flow Rate: 2 ml/min.

Detection: UV at 254 nm.

System 6

HPLC-EICD

This system is applicable for the determination of morphine in cerebrospinal fluid and plasma in levels as low as 1 ng/ml in 0.1 to 0.4 ml of these fluids. It is also suitable to determine the pharmacokinetics of morphine in biological fluids (253).

Column:  $\mu$ -Bondapak C<sub>18</sub>, 300 mm x 4 mm ID, used in conjunction with an amperometric detector system. A guard column packed with corasil-C<sub>18</sub> is placed before the reverse-phase column.

Mobile Phase: Isocratic composed of 0.07 M KH<sub>2</sub>PO<sub>4</sub> containing 0.5 m MEDTA and modified with 5% acetonitrile and 8% methanol.

Flow-Rate: 1.0 ml/min.

Detection: Electrochemical detector.

System 7

The following system 7 has been reported for the analysis of poppy straw concentrate (254).

Column: Phenyl Bondapak, 25 cm x 5 mm ID, it is used in conjunction with a 7 cm x 2 mm ID, guard column packed with C<sub>18</sub>/Corasil, particle size 37-50 µm.

Mobile Phase: Solvent A is acetonitrile-water (5:95) and solvent B is acetonitrile-water (20:80). Both solvents contained 1 ml/l of glacial acetic acid and 0.04 ml/l of N,N-dimethyloctylamine. The pH of both is adjusted to 3.5 with sodium hydroxide solution.

Flow Rate: 1.0 ml/min.

Detection: UV absorption at 275 nm.

System 8

The following system 8 has been reported for the routine determination of morphine and other alkaloids in gum opium (255).

Column: Nucleosil-IOCN

Mobile Phase: 1% Ammonium acetate buffer solution (adjusted to pH 5.8 with acetic acid)-acetonitrile-dioxan (8:1:1).

Flow Rate: 1.5 ml/min.

Detection: UV at 254 nm.

System 9  
HPLC-cation  
exchange

This system is described for the qualitative detection of contaminants including morphine in illicit diamorphine seizures (256).

Column: Zipax SCX, 12 cm x 2.1 mm ID.

Mobile Phase: 0.2 M H<sub>3</sub>BO<sub>3</sub> (adjusted to pH 9.3 with 40% NaOH)-acetonitrile-n-propanal

(86:12:2). The phase is adjusted to pH 9.8 with NaOH (40% aqueous).

Flow Rate: 2 ml/min.

Detection: UV absorption at 270 nm.

System 10  
HPLC-EICD

The following system is employed to precisely determine morphine levels as low as 1 ng/ml in 1-2 ml of serum without the need for derivatization, (257).

Column: Reverse phase containing octa decylsilane (monomeric) bonded to Lichrosorb (silica), 30 cm x 4 mm ID.

Mobile Phase: Isocratic consisting of 85% methanol and 15% 0.01 M  $\text{KH}_2\text{PO}_4$  buffer.

Flow Rate: 1.0 ml/min.

Detection: Electrochemical detector operated at an applied voltage of +1000 mV.

System 11

The following system has been applied for the detection of morphine, codeine, thebaine and papaverine (258).

Column: Nova-Pak  $\text{C}_{18}$  Radial Pak, 10 cm x 8 mm ID.

Mobile Phase: A) 10 m M Potassium perchlorate, monobasic; 5 m M n-butylamine, (pH 3.0 adjusted with perchloric acid)  
B) Acetonitrile

Flow Rate: 3 ml/min.

Detection: UV at 280 nm.

System 12

This system has been reported for the quantitative determination of morphine in Paregoric USP (259).

Column:  $\mu$ -Bondapac/ $\text{C}_{18}$ , 30 cm x 4 mm ID, it consists of monomolecular layers of octadecyltrichlorosilane permanently bonded to silica via silicon-carbon bonds.

Mobile Phase: 0.1 M K H<sub>2</sub>PO<sub>4</sub> buffer solution in 7% (v/v) methanol in water.

Flow Rate: 1.8 ml/min. (inlet pressure approximately 2100 psig).

Detection: UV at 254 nm.

System 13

The following system has been reported for the determination of morphine, codeine and dionin, and is applied for quantitation of codeine in syrups using ion pair formation (260).

Column: A microparticulate octadecylsilan  $\mu$ -Bondapak C<sub>18</sub>, 30 cm x 4 mm ID, and a microparticulate octasilane, 25 cm x 4.6 mm ID.

Mobile Phase: Contains 0.005 M pairing ion and 0.01 M ammonium nitrate in acetonitrile-water (375:625). After mixing, it is adjusted to pH 3.3 with acetic acid and filtered through a 5- $\mu$ m filter.

Flow Rate: 2-3 ml/min.

Detection: A UV detector (254 nm).

System 14

This system has been reported for the analysis of morphine, codeine, thebaine and for several synthetic related derivatives (morphinane derivatives) (261).

Column:  $\mu$ -Bondapak C<sub>18</sub>.

Mobile Phase: Methanol-Water (40:60), containing 0.005 M n-heptanesulfonic acid.

Flow Rate: 1.2 ml/min.

Detection: UV absorption at 254 nm.

- System 15      The following system is applicable for the separation of heroin and its hydrolysis products including morphine. It is also applied to study the kinetics of heroin hydrolysis (262).
- Column:      Fully porous 10- $\mu$ m silica particles bonded with a monomolecular layer of octadecylsilane ( $\mu$ -Bondapak C<sub>18</sub>), 30 cm x 3.9 mm ID.
- Mobile Phase:      Acetonitrile-0.015 M potassium dihydrogen phosphate adjusted to pH 3.5 with 2 N phosphoric acid (3:7 v/v).
- Flow Rate:      1 ml/min.
- Detection:      UV absorption at 235 nm.
- System 16      This system has been employed to detect morphine in human plasma (263). Morphine was extracted from with a Bond-Elut ODS cartridge, elution being effected with methanol. The eluate was evaporated to dryness and the residue dissolved in the mobile phase.
- Column:      Hypersil ODS (10 cm x 4 mm ID) equipped with a guard column of same material.
- Mobile Phase:      0.1 M heptanesulfonic acid - 1% ammonium acetate solution - 2% H<sub>2</sub>SO<sub>4</sub> - 25% acetonitrile (6:5:2:87).
- Flow Rate:      1 ml/min.
- Detection:      Electrochemical.
- Many other HPLC for morphine have been also reported (264-280).

## 9.8 Immunoassays

### 9.8.1 Radioimmunoassays (RIA)

The RIA for morphine was first described by Spector and Parker (281) and then by Spector (282).

Antibodies to morphine were obtained by immunizing rabbits with a *3-O-carboxymethylmorphine-bovine serum albumin conjugate*; inhibition of binding of marker ( $[^3\text{H}]$ -dihydromorphine) was obtained at concentrations of morphine and its surrogates of less than 1 ng/ml. Antibody-bound  $[^3\text{H}]$ -dihydromorphine was separated from unbound marker by precipitation with ammonium sulfate and counted by liquid scintillation (281,282). The assay was applicable to the measurement of opiates in unextracted serum. The absolute sensitivity of the RIA in serum was 50 to 100 pg in a final assay mixture of 0.5 ml (282).

- Antibodies to morphine have subsequently been prepared by conjugation through the OH group at C<sub>6</sub> of the morphine molecule such as *morphine 6-hemisuccinate* or through an *azo linkage* at C<sub>2</sub> (283,284).

Antibodies to morphine C<sub>6</sub> - hemisuccinate bind morphine 3-glucuronide, an *in vivo* conjugation product of morphine, almost as effectively as morphine itself; in contrast to antibodies to C<sub>3</sub> carboxymethylmorphine, which display a marked decrease in binding affinity for molecules of morphine which contain the hydrophilic glucuronide group (284).

Binding measurements similar to those used in the morphine immunoassay have been used to detect antibodies to morphine in serum of heroin addicts (285).

A sensitive and rapid RIA for morphine and immunologically related substances in urine and serum has been described (286).

- The RIA has been used to characterize the pharmacokinetic profile of morphine (287-289). Caltin (290) however, has questioned the validity of RIA for the pharmacokinetic analysis, demonstrating a variability in the specificity of the antibody and interference from morphine metabolites, that can result in discrepant interpretation.



Stanski et al (291) have compared morphine concentration determined by RIA and GLC, demonstrating a 27% overestimation of the true morphine concentration by RIA at time intervals 1 hour after drug administration and concluding that the RIA was significantly less precise than the GLC assay.

In spite of the above facts, RIA still popular technique, it has been employed for quantitative determination of morphine in capsules of *Papaver somniferum* (292):

Morphine is extracted from poppy capsules and separated from codeine and thebaine.

The assay involves incubation of an aqueous extract with antiserum (raised in rabbits against the morphine 3-hemisuccinate-bovine serum albumin conjugate).  $^3\text{H}$ -labeled morphine and phosphate buffer saline, separation of free and antibody bound  $^3\text{H}$  by treatment with activated charcoal and centrifugation and counting the radioactivity of the supernatant phase by a liquid scintillation technique.

The calibration graph is rectilinear for 0.01 to 0.2 ng of morphine.

A simple RIA for morphine in human plasma has recently described (293) in which commercially available antiserum and solid phase second antibody are used in conjunction with  $^{125}\text{I}$  labeled morphine. Labeling is effected by mixing a solution ( $1\text{ mg/ml}^{-1}$ ) of 1,3,4,6-tetrachloro-3,6-diphenylglycouril in  $\text{CH}_2\text{Cl}_2$  with  $\text{Na }^{125}\text{I}$  and morphine sulfate pentahydrate in phosphate buffer (pH 7.5), the derivative is cleaned up in an octadecylsilica Bond-Elut cartridge. It has been noted that cross reactivity with morphine metabolites is negligible. This method can be used for morphine pharmacokinetics in man after IV administration (293).

*In summary RIA are capable of detecting picogram amounts of morphine, but they lack specificity as they cannot distinguish between morphine and some structurally related substances.*

### 9.8.2 Free Radical Assay Technique (FRAT)

In this technique a stable free radical, a nitroxide, is attached to morphine (294). The free radical contains unpaired electrons which may be observed and measured by an electron spin resonance (ESR) spectrometer (295). *This instrument measures the energy absorbed by unpaired electrons as they flip from a stable to a metastable position in a magnetic field.*

- The FRAT system was developed for the detection of morphine in urine. It is still successfully used for this purpose:-

A drop of the sample is mixed with a drop of antibody and nitroxide-labeled morphine in a capillary tube, the tube is then inserted in the ESR spectrometer and the resulting signal is measured.

The FRAT procedure has been extended to other drugs such as amphetamine, methadone and barbiturates.

FRAT sensitivity is not the highest available but it is usually more than adequate for most applications (296).

### 9.8.3 Haemagglutination Inhibition

In this technique the label to which the drug molecules are attached is a red blood cell which has been stabilized by treatment with formaldehyde (296). Since antibodies are bivalent, they may bind more than one red blood cell and agglutination occurs. When the test sample contains antigens, which compete for the active sites on the antibody, agglutination is inhibited (296).

Morphine can be assayed by this technique (297,298):-

Tanned erythrocytes sensitized with a 3-carboxymethylmorphine conjugate were agglutinated by anti-morphine antibody, with inhibition of the agglutination by morphine, codeine and heroin (297).

- Methodology and application of this technique for the detection of morphine have been reported (298).

*This assay is suitable for the rapid screening of large number of samples. It is less quantitative than the RIA (299).*

#### 9.8.4 Enzyme Mediated Immunoassay Technique (EMIT)

In this technique, the label attached to the drug here is the *enzyme lysozyme*. This enzyme catalyses hydrolysis of the mucopolysaccharide components in the cell walls of certain bacteria. When the antibody binds morphine attached to lysozyme, the enzyme is inactivated. In the presence of drug, the enzyme is released and hydrolysis takes place and can be measured (296,300). Rubenstein et al (300) were the first to apply this technique to morphine. Several others have also applied this technique (300-308).

EMIT has been applied for the analysis of drugs (including morphine) in *blood, bile and tissues* (304). Morphine levels determined in blood by EMIT correlated well with those attained by a fluorometric technique (304).

Urine samples were examined for drugs by TLC and by EMIT, the results of both methods were compared for the detection of opiates, barbiturates and amphetamines. In more than 90% of the determinations, identical results were obtained with both methods (305).

Morphine can be quantitatively determined by this technique (206).

- A combined method utilizing EMIT-LCEC (liquid chromatography with electrochemical detection) for the identification, confirmation and quantitation of opiates in biological fluids has been described (307):-

EMIT was used to test for opiates (morphine, hydromorphone and codeine) in extracts of *blood, bile and tissue homogenates*, all opiate positive specimens were then analyzed by HPLC with electrochemical detection. Blood specimens were quantitated by the HPLC procedure which involved a column (25 cm x 4.6 mm ID) of ultrasphere ODS (5 $\mu$ m) and 50 mM phosphate buffer (pH 6) - methanol (3:2) as the mobile phase.

The limit of detection (as morphine) of the immunoassay technique was:

0.02 mg l<sup>-1</sup>, 0.2 mg l<sup>-1</sup> and 0.1 mg Kg<sup>-1</sup> for blood, bile and tissue homogenates respectively. Response for HPLC method rectilinear from 0.005 to 0.3 mg l<sup>-1</sup> for the three opiates with detection limits of 0.005 mg l<sup>-1</sup> for each (307).

- A modification of the technique has been also reported (308).

## 9.9 Other Quantitative Methods

### 9.9.1 Isotope Dilution

A method was developed for quantitative determination of morphine in opium based on the isotope dilution technique (309). Morphine-2-<sup>3</sup>H and morphine-N-<sup>14</sup>CH<sub>3</sub> are used as radioactive standards. A mixture of opium and the radioactive morphine standard is triturated with dimethyl sulfoxide, dispersed on diatomaceous earth and acidic aluminum oxide, and suspended in water. The aqueous suspension is transferred to a chromatographic column of acidic aluminum oxide, and the alkaloids are eluted with water. Alternatively, the mixture of opium and radioactive morphine is triturated with a little water and dispersed on diatomaceous earth, and the alkaloid bases are liberated with ammonia. The powder mixture is transferred to a column of neutral aluminum oxide and eluted with chloroform-isopropyl alcohol (3:1). Phenolic and non-phenolic alkaloids are separated by extraction at pH 13, and morphine crystallizes from the aqueous phase after adjustment of pH to 9. The crystals are collected and recrystallized to constant radioactivity.

- Determination of radioactive-labeled morphine, codeine, dihydromorphine and their metabolites in biological materials has been reported (310):-

#### *For free morphine*

The sample (1 ml) is mixed with water (1 ml), 0.1% non-labeled morphine sulfate solution 0.5 ml and 40% K<sub>2</sub>HPO<sub>4</sub> solution (2 ml). The resulting mixture is shaken for 30 minutes with 30% pentanol solution in 1,2-dichloroethane (15 ml) and centrifuged. The organic phase is washed with 4% K<sub>2</sub>HPO<sub>4</sub> solution (5 ml). An aliquot (10 ml) of the organic phase is evaporated to dryness, dissolved in pentanol (0.8 ml) and to this counting solution is added (10 ml). The radioactivity is then measured with a scintillation counter.

#### *For total morphine*

The sample has to be hydrolysed with HCl by heating in an autoclave and neutralized with sodium bicarbonate solution before treatment as free morphine.

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# ANALYTICAL PROFILE OF PAPAVERINE HYDROCHLORIDE

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## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

- 1-[(3,4-Dimethoxyphenyl) methyl]-6,7-dimethoxy isoquinoline hydrochloride.
- Isoquinoline, 1-[(3,4-dimethoxyphenyl) methyl]-6,7-dimethoxy, hydrochloride.
- 6,7-Dimethoxy-1-veratrylisoquinoline hydrochloride.
- 6,7-Dimethoxy-1-(3,4-dimethoxybenzyl) isoquinoline hydrochloride.

#### 1.1.2 Generic Name

Papaverine Hydrochloride.

#### 1.1.3 Trade Names

Artegodan; Cardover; Cerebid; Cerespan; Diapav; Kavrin; Myobid; P-200; Panergon; Papacon; Papital T.R.; Pava-2 Caps; Pavabid; Pavacap; Pavacen; Pavaclar; Pavadel; Pavagen; Pavagrant; Pavakey; Pavased; Pavastet; Pavatran; Pava-Wol; Pavine TD; Quabid; Sustaverine; Therapav; Vasal; Vasocap; Vaso-Pav, Vasospan.

### 1.2 Formulae

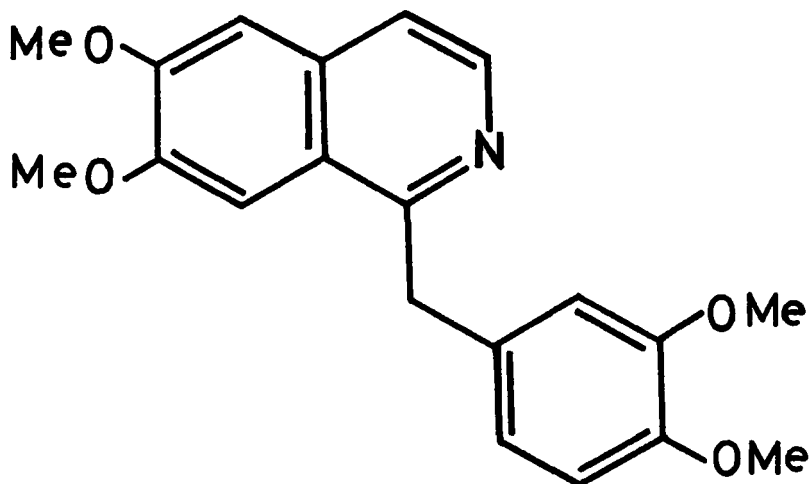
#### 1.2.1 Empirical

$C_{20}H_{21}NO_4$  (Papaverine)

$C_{20}H_{22}Cl NO_4$  (Papaverine Hydrochloride)

#### 1.2.2 Structural

Papaverine is a benzyloisoquinoline alkaloid. The structure of which was established by Goldschmiedt and Co-Workers in 1883-1888 (1,2) and have been confirmed by the total synthesis of papaverine which was achieved by several authors (3-9).



Papaverine is optically inactive alkaloid.  
It does not contain any chiral center (10).

#### 1.2.3 CAS Registry Number

Papaverine [58-74-2]

Papaverine Hydrochloride [61-25-6]

#### 1.2.4 Wiswesser Line Notations

T66 CNJ B1R C O1 DO1 & HO1  
\_101 (Papaverine).

T66 CNJ B1R CO1 DO1 & HO1  
\_101 & GH (Papaverine HCl) (11 ).

#### 1.3 Molecular Weight

339.38 (Papaverine)

375.84 (Papaverine HCl)

#### 1.4 Elemental Composition

C, 70.78%; H, 6.24%; N, 4.12%;  
O, 18.86% (Papaverine).

C, 63.91%; H, 5.90%; Cl, 9.43%;  
N, 3.73%; O, 17.03% (Papaverine HCl).

### 1.5 Appearance, Color, Odor and Taste

Papaverine crystallizes as rhombic prisms from ethanol-ether mixture or colorless needles from chloroform-petroleum ether mixture. Odorless and it has a bitter taste (12).

Papaverine hydrochloride crystallizes as monoclinic rods from water (13).

It may occur as white or almost white crystals or a white or almost white crystalline powder (14).

Odorless and has a bitter saline taste.

### 1.6 Loss on Drying

When papaverine hydrochloride dried at 105° to constant weight, it loses not more than 1.0% of its weight (15).

## 2. Physical Properties

### 2.1 Melting Range

Papaverine melts at 147°. It sublimes at 135-140° at 11 mm pressure and 2 mm distance (13).

Papaverine hydrochloride melts in the range 220-225° (13).

### 2.2 Eutectic Temperature

The eutectic temperature of papaverine hydrochloride is recorded as follows (16):-

	Hot Bar Method	Microscope Hot Stage
Sal.	165°	165°
Dic.	157°	155°

---

Sal. = acetaminosalol, Dic. = dicyandiamide

### 2.3 Solubility

-Papaverine is almost insoluble in water, sparingly soluble in cold ethanol, soluble in chloroform, hot ethanol, acetone and hot benzene (13).

-One gram of papaverine hydrochloride is soluble in 40 ml of water, in 120 ml of ethanol (96%), in 10 ml chloroform and practically insoluble in ether (15).

## 2.4 pH Range

pH of a 2% w/v solution of papaverine HCl is 3.0 to 4.5 (15).

pH of 0.05 molar solution (HCl salt) is 3.9 (13).

## 2.5 Dissociation Constant

pK<sub>a</sub> (papaverine hydrochloride) = 6.4(25°)

A spectrophotometric determination of the dissociation constant of papaverine gives average value of pK 7.60 (17).

## 2.6 Crystal Structure

The crystal structure of papaverine and papaverine hydrochloride have been determined by X-ray diffraction technique (18).

The space group of papaverine was found to be  $P2_12_12_1$  with  $a = 9.50\text{\AA}$ ,  $b = 29.22\text{\AA}$ ,  $c = 6.35\text{\AA}$ .

The space group of papaverine hydrochloride as  $P2_1/c$  with  $a = 13.10\text{\AA}$ ,  $b = 15.80\text{\AA}$ ,  $c = 9.22\text{\AA}$ ,  $\beta = 92^\circ 36'$ .

The cell dimensions were obtained from rotation and Weissenberg photographs and  $\beta$  (for the hydrochloride) from goniometric measurements and b-axis zero-level Weissenberg films (18).

## 2.7 X-ray Powder Diffraction

The X-ray diffraction pattern of papaverine hydrochloride was determined with a Philips X-ray diffraction spectrogoniometer equipped with PW 1730 generator. X-ray radiation was provided by a copper target (Cu anode 2000 w). High intensity X-ray tube operated at 40 KV and 20 mA was used. The monochromator was a single curved crystal one ( $\gamma$ -1.5418Å°).

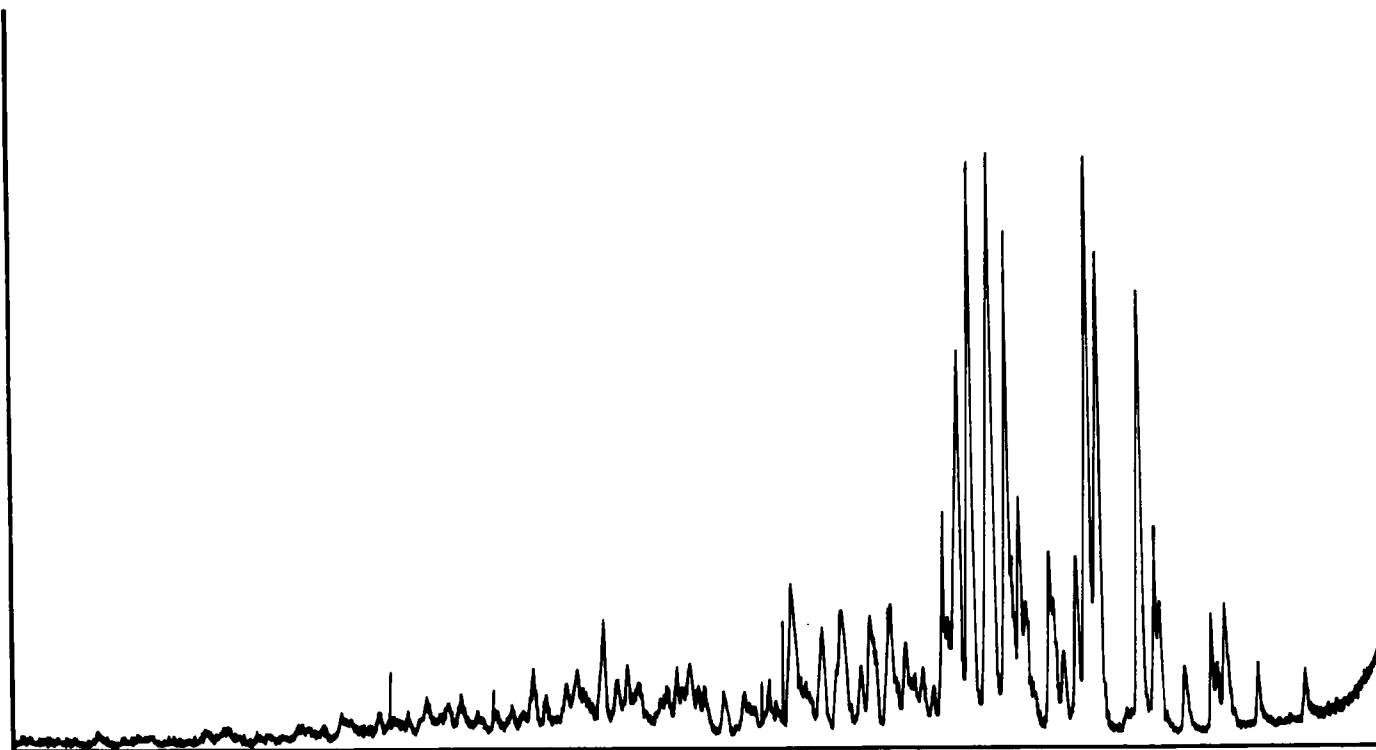
The unit was also equipped with Philips PM 8210 printing recorder and digital printer. Divergence slit and the receiving slit were 1°. The scanning speed of the goniometer used was 0.02 2 $\theta$ /sec., recorder full scale was 10,000 counts at recorder speed of 4 mm/2 $\theta$ . The lower level and upper one of the signal control were 35 and 75 respectively.

The X-ray pattern of papaverine hydrochloride is presented in Fig. 1. Interplanar distance and relative intensity are tabulated in table 1.

Table 1 : X-Ray Powder Diffraction Pattern of Papaverine HCl

d(Å°)	I/I <sub>0</sub>	d(Å°)	I/I <sub>0</sub>	d(Å°)	I/I <sub>0</sub>
10.10	13	3.44	99	2.20	15
7.92	14	3.36	68	2.11	13
6.89	23	3.27	40	2.08	16
6.69	15	3.22	12	2.06	13
6.55	23	3.15	15	2.03	24
5.97	13	3.11	13	1.97	15
5.47	25	3.06	19	1.95	13
5.38	37	2.98	25	1.88	15
5.14	77	2.90	17		
4.59	83	2.84	15		
4.46	99	2.76	24		
4.36	33	2.75	12		
4.21	17	2.68	21		
4.11	25	2.61	13		
3.87	25	2.56	29		
3.82	43	2.48	13		
3.71	88	2.26	12		
3.59	100	2.24	16		

d=interplanar distance, I/I<sub>0</sub>=relative intensity (based on highest intensity of 100).



**FIG. 1 : THE X-RAY DIFFRACTION PATTERN OF PAPAVERINE HCL.**



## 2.8 Spectral Properties

### 2.8.1 Ultraviolet Spectrum

The UV spectrum of papaverine hydrochloride in methanol was scanned from 200 to 400 nm (Fig. 2) using DMS 90 Varian spectrometer. Papaverine hydrochloride exhibited the following UV data (Table 2).

Table 2 : UV Characteristics of Papaverine Hydrochloride

<u><math>\lambda</math> max. nm</u>	<u>log <math>\epsilon</math></u>	<u>A(1%, 1 cm)</u>
238.5	4.81	1725
283	4.13	361
312	4.12	356
325	4.08	326

Other reported UV spectral data for papaverine in ethanol  $\lambda$  max. nm 327(5012), 314(3981), 280(7943), 238(63096) (11), for papaverine hydrochloride  $\lambda$ max. nm 250 (E 1%, 1 cm 1830); 284 (E 1%, 1 cm 193) and 310 (E 1%, 1 cm 253). (19,20).

### 2.8.2 Infrared Spectrum

The IR spectrum of papaverine hydrochloride as KBr-disc was recorded on a Perkin Elmer 580B. Infrared spectrophotometer to which an infrared data station is attached (Fig. 3). The structural assignments have been correlated with the following frequencies (Table 3).

Table 3 : IR Characteristics of Papaverine Hydrochloride

<u>Frequency <math>\text{cm}^{-1}</math></u>	<u>Assignment</u>
3020,2965,2940	CH (stretch)
2845	-OCH <sub>3</sub>
1635	C=N-
1520,1510,1485	C=N (in conjugated cyclic system)
1610,1595	C=C (aromatic)

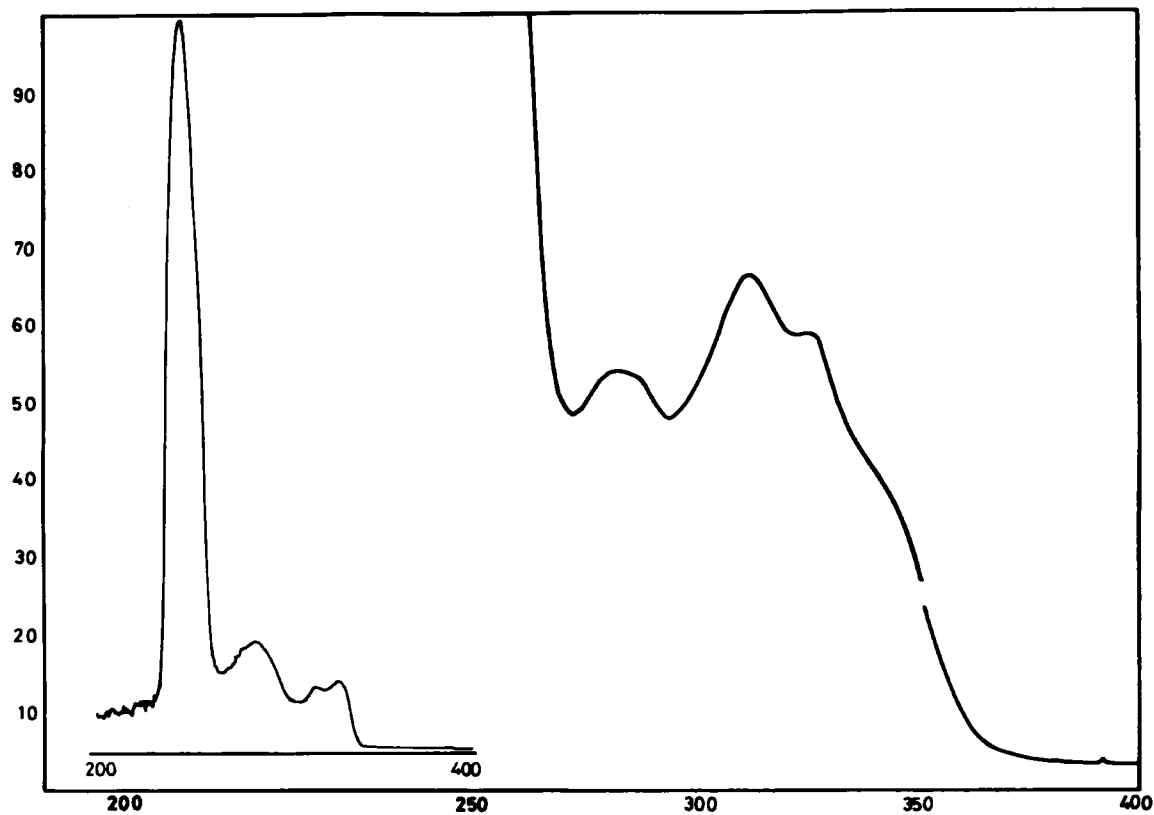


FIG. 2 : THE UV SPECTRUM OF PAPAVERINE HCL.

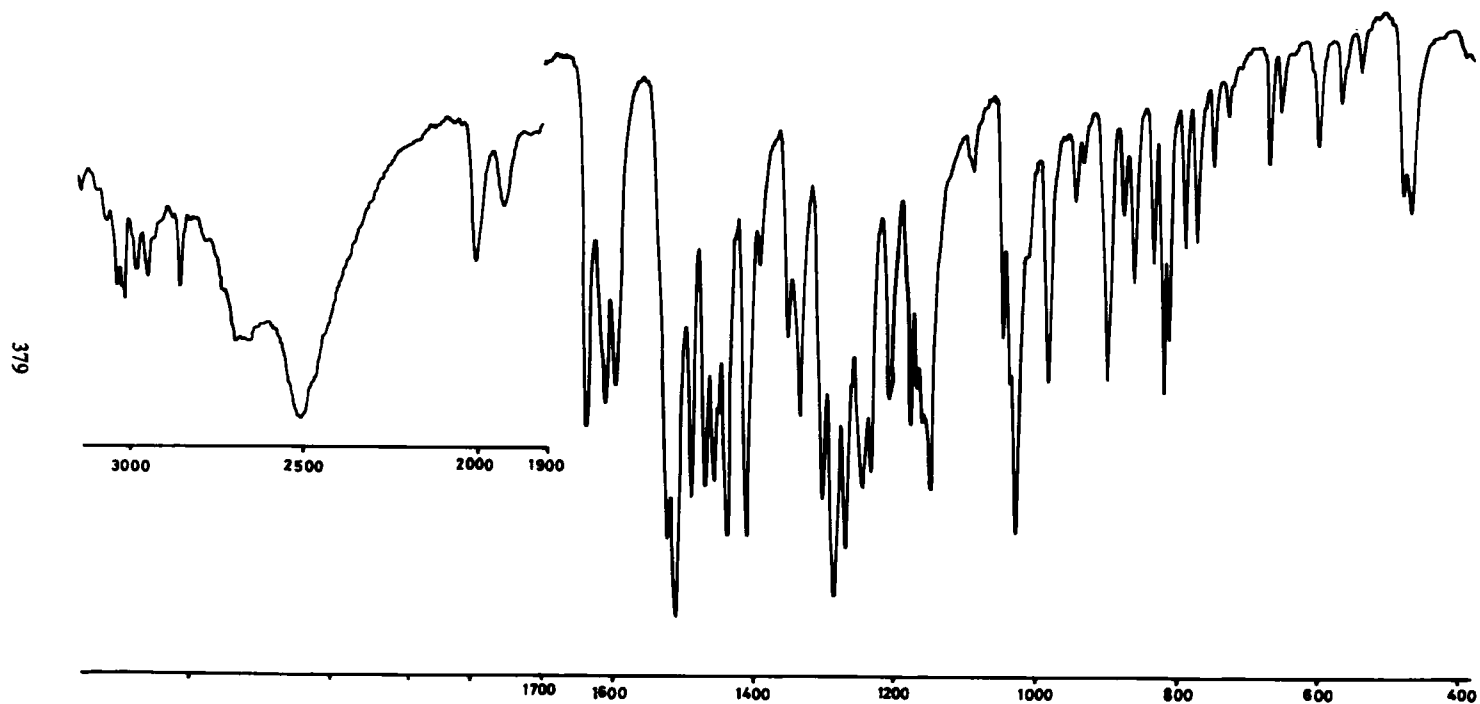


FIG. 3 : THE IR SPECTRUM OF PAPAVERINE HCL AS KBR DISC.

The IR of papaverine HCl exhibited the following other characteristic bands:-

3000, 2500, 1995, 1955, 1465, 1455, 1435, 1408, 1350, 1330, 1300, 1280, 1265, 1245, 1232, 1205, 1175, 1148, 1045, 1028, 980, 940, 895, 870, 860, 830, 820, 810, 790, 770, 745, 720, 665, 650  $\text{cm}^{-1}$ .

Other I.R. data for papaverine and papaverine HCl have been reported (11, 19, 20).

### 2.8.3 Nuclear Magnetic Resonance

#### 2.8.3.1 $^1\text{H}$ -NMR Spectra

The  $^1\text{H}$ -NMR spectra of papaverine hydrochloride in  $\text{CDCl}_3$  (Fig. 4) and papaverine in  $\text{DMSO-D}_6$  (Fig. 5) were recorded on a Varian FT 80A, 80 MHz NMR spectrometer using TMS as an internal reference in both. The following structural assignments have been made (Table 4).

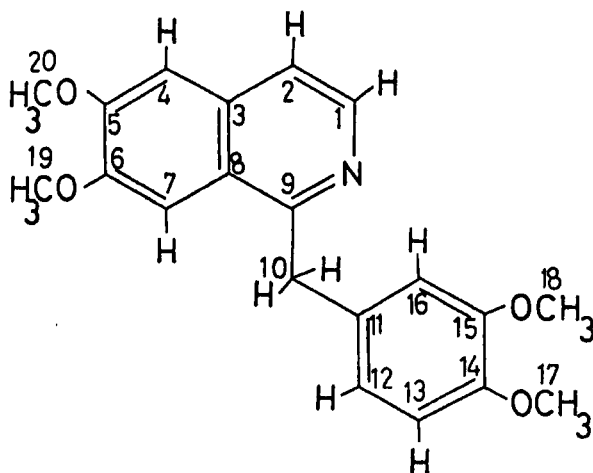


Table 4.  $^1\text{H}$ -NMR Characteristics of Papaverine

Group	Chemical Shift (ppm)	
	Papaverine HCl ( $\text{CDCl}_3$ )	Papaverine ( $\text{DMSO-D}_6$ )
$\text{H}_2$	aromatic protones	8.28 (d)
$\text{H}_1, \text{H}_4$	absorb as a group	7.54 (d)

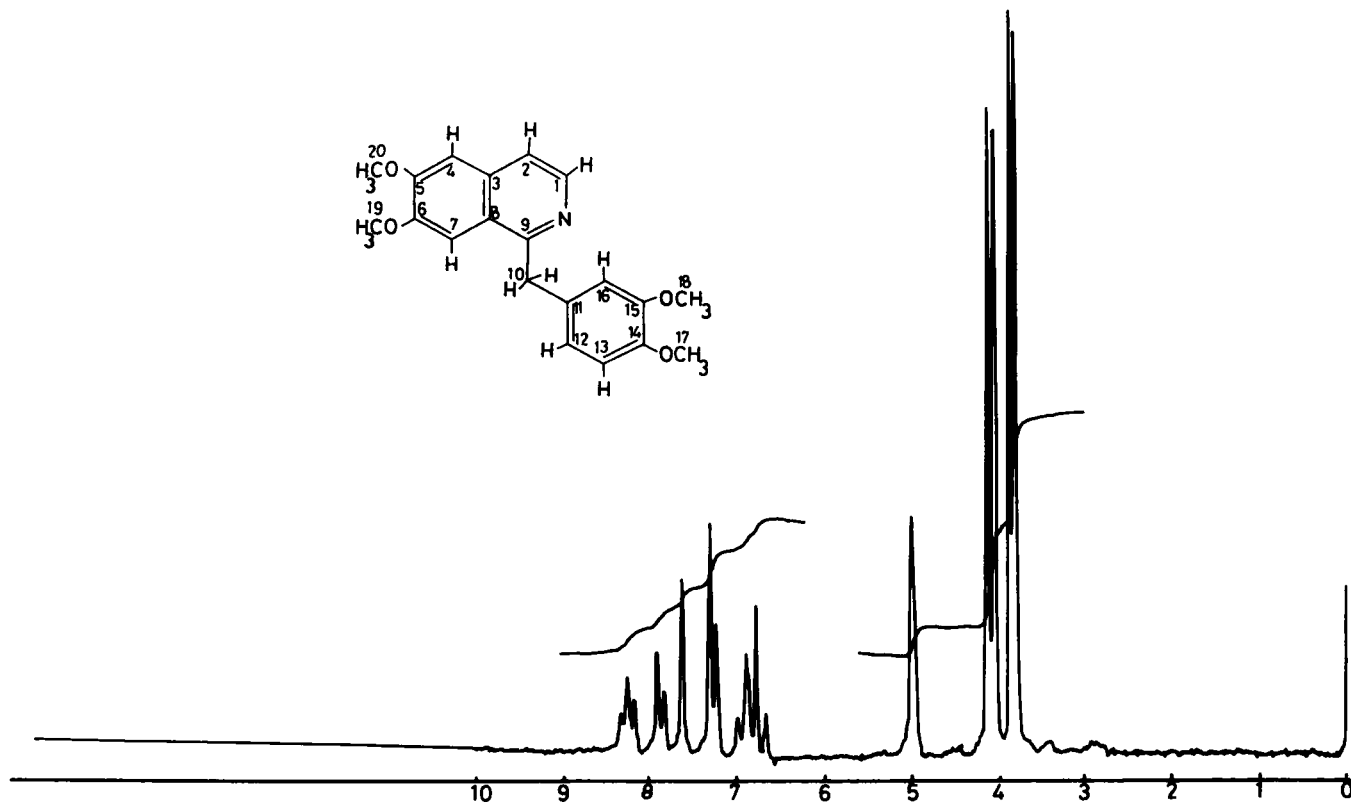


FIG. 4 : THE  $^1\text{H}$ -NMR SPECTRUM OF PAPAVERINE HCL IN  $\text{CDCl}_3$

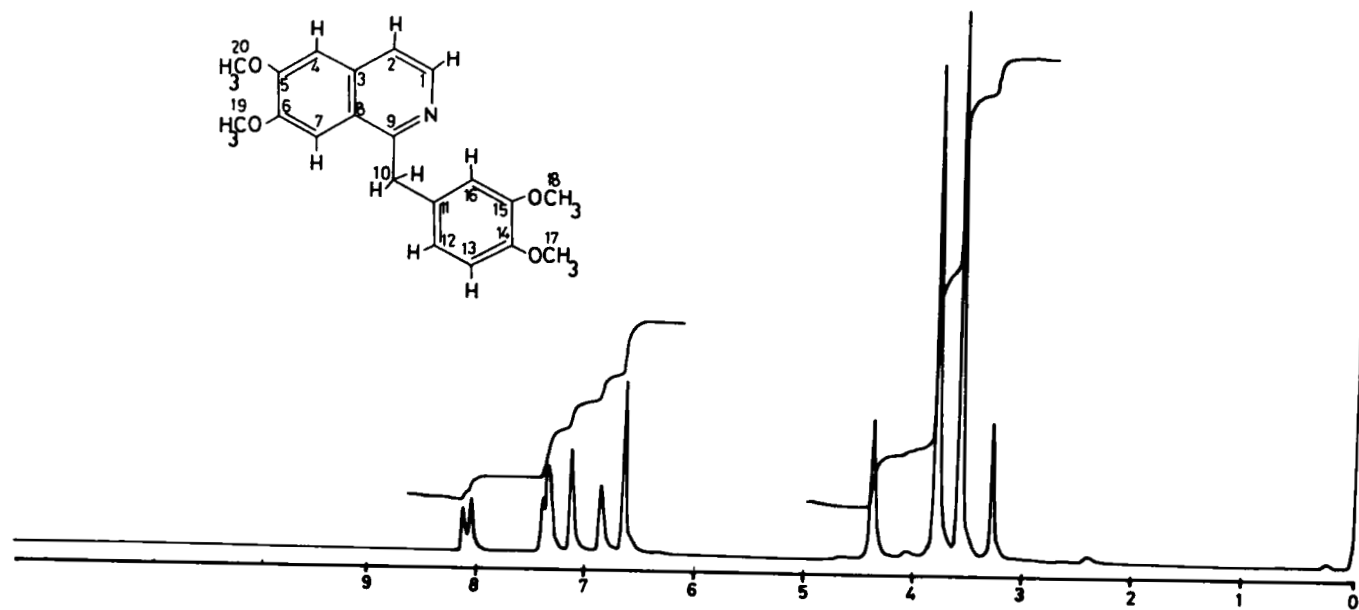


FIG. 5 : THE  $^1\text{H}$ -NMR SPECTRUM OF PAPAVERINE IN  $\text{DMSO}-d_6$

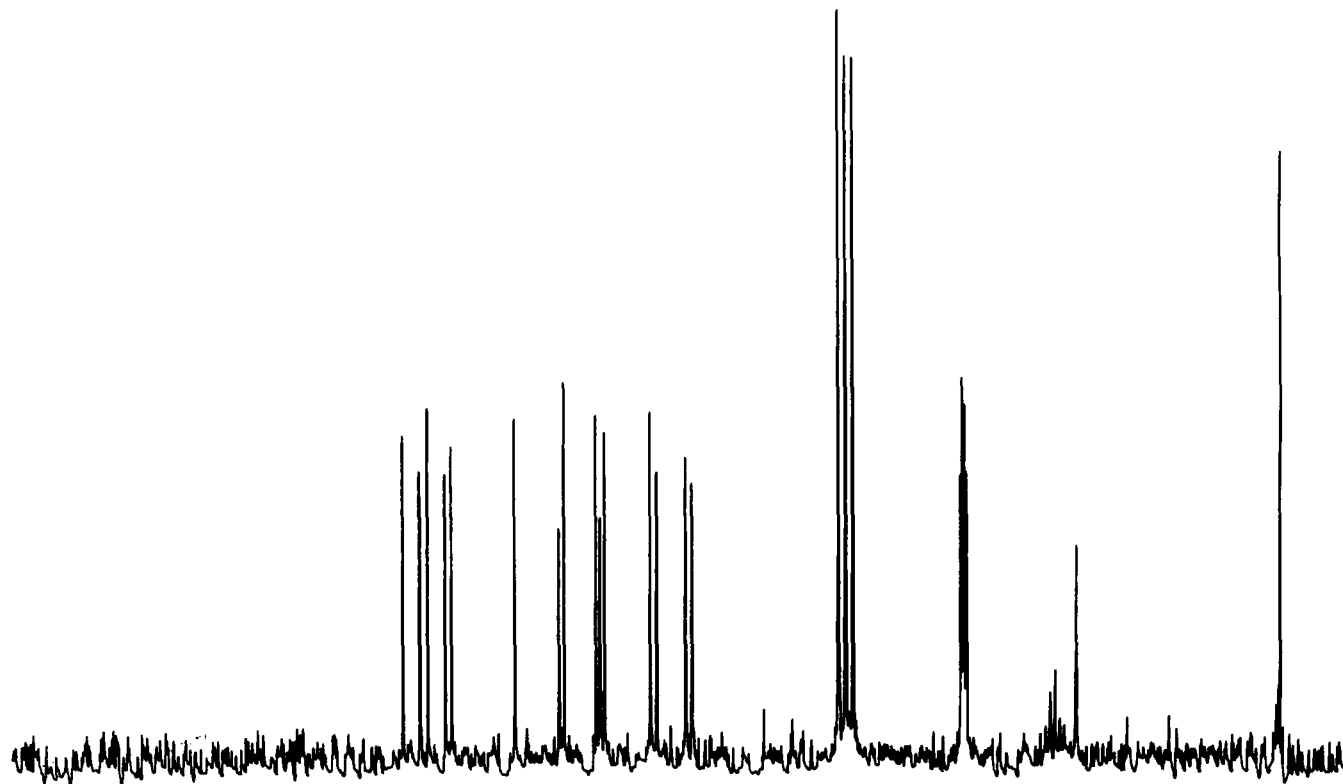


FIG. 6 : THE  $^{13}\text{C}$  - NMR COMPLETELY DECOUPLED SPECTRUM OF PAPAVERINE HCL.

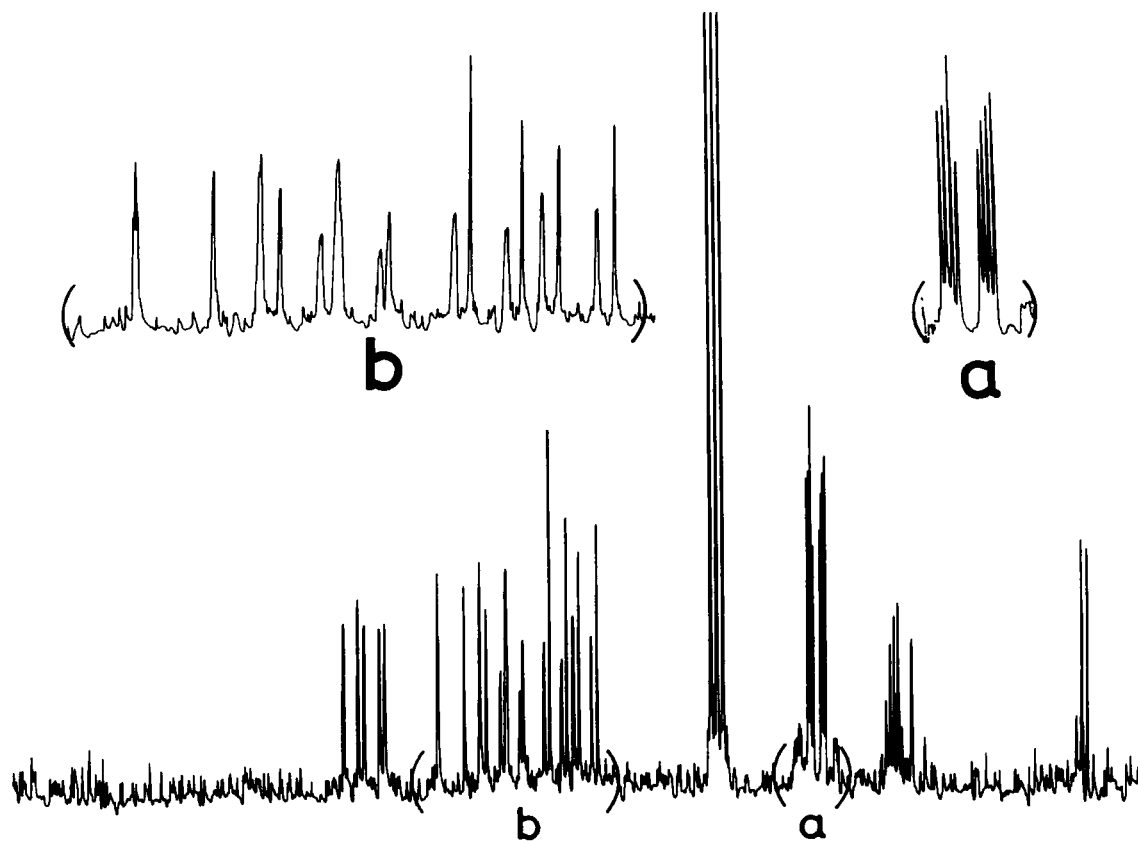


FIG. 7: THE  $^{13}\text{C}$ -NMR OFF RESONANCE SPECTRUM OF PAPAVERINE HCL.



Group	Chemical Shift (ppm)	
	Papaverine HCl (CDCl <sub>3</sub> )	Papaverine (DMSO-D <sub>6</sub> )
H <sub>7</sub>	of signals between 6.8.26 and 6.60 and integrating for 7 protons	7.31 (s)
H <sub>16</sub>		7.03 (s)
H <sub>12</sub> , H <sub>13</sub>		6.82 (s)
2 H <sub>10</sub>		4.48 (s)
2 OCH <sub>3</sub> 19, 20	4.05(s), 3.99(s)	3.90 (s)
2 OCH <sub>3</sub> 17, 18	3.80(s), 3.75(s)	3.68,3.87 (2s)

s = singlet, d = doublet, m = multiple.

Other <sup>1</sup>H-NMR data for papaverine has been reported (11,21-23).

#### 2.8.3.2 <sup>13</sup>C-NMR Spectra

The <sup>13</sup>C-NMR completely decoupled and off-resonance spectra of papaverine hydrochloride were recorded over 5000 Hz using a Joel FX-100 NMR spectrometer. The alkaloidal salt was dissolved in CDCl<sub>3</sub> using a 10 mm sample tube and TMS as internal standard at ambient. The spectra are presented in Fig. 6 and Fig. 7.

The carbon chemical shifts were assigned on the basis of the additivity principles and off-resonance splitting pattern (Table 5).

Table 5. Carbon Chemical Shifts of Papaverine

Carbon	Chemical Shifts [ppm]	Carbon	Chemical Shifts [ppm]
C <sub>1</sub>	153.93 (d)	C <sub>5</sub>	152.53 (s)
C <sub>2</sub>	104.97 (d)	C <sub>6</sub>	149.59 (s)
C <sub>3</sub>	128.07 (s)	C <sub>7</sub>	106.87 (d)
C <sub>4</sub>	111.37 (d)	C <sub>8</sub>	129.45 (s)

Carbon	Chemical Shifts [ppm]	Carbon	Chemical Shifts [ppm]
C <sub>9</sub>	156.93 (s)	C <sub>15</sub>	136.91 (s)
C <sub>10</sub>	40.09 (t)	C <sub>16</sub>	120.82 (d)
C <sub>11</sub>	122.40 (s)	C <sub>17</sub>	56.18 (q)
C <sub>12</sub>	121.64 (d)	C <sub>18</sub>	55.83 (q)
C <sub>13</sub>	119.48 (d)	C <sub>19</sub>	56.53 (q)
C <sub>14</sub>	148.24 (s)	C <sub>20</sub>	56.83 (q)

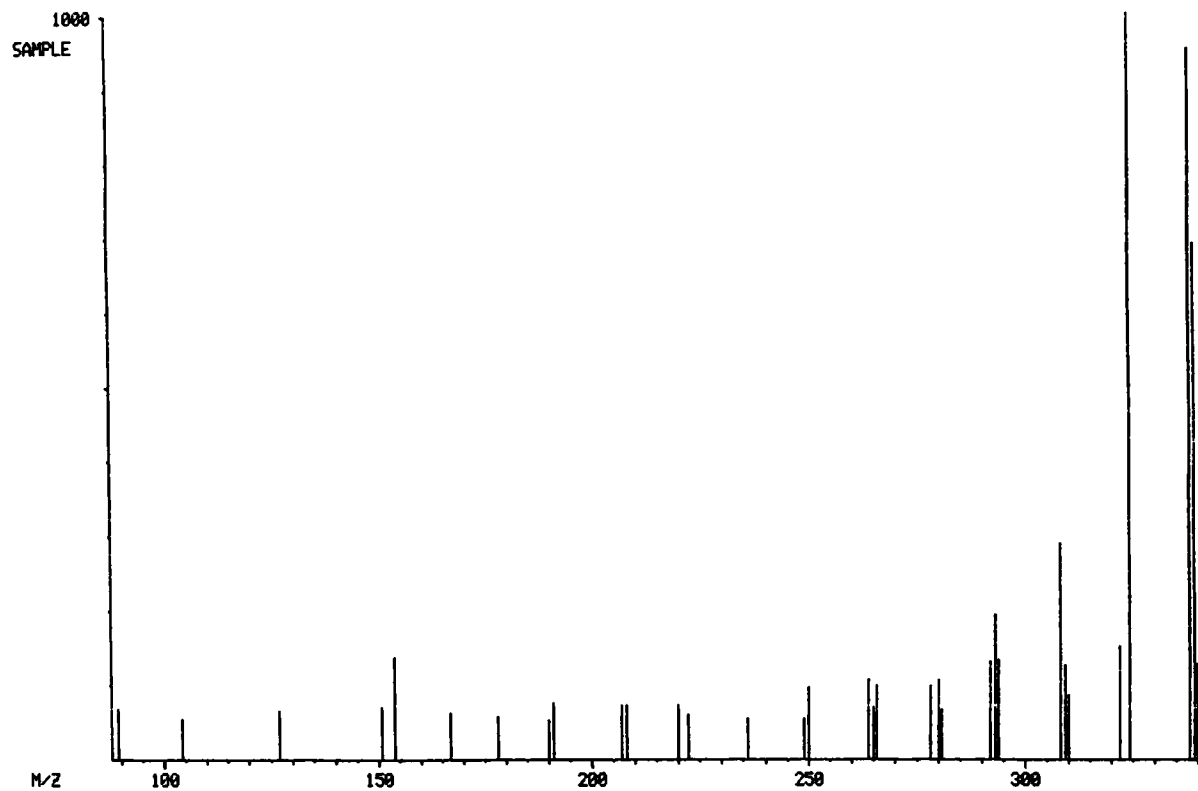
s = singlet; d = doublet; t = triplet; q = quartet

#### 2.8.4 Mass Spectrum

The mass spectrum of papaverine is presented in Fig. 8. This was obtained by electron impact ionization on a Finnigan-Mat 5100 GC/MS system. The spectrum was scanned to mass 350 amu. It shows a molecular ion peak  $M^+$  at  $m/e$  339. The base peak is at  $m/e$  324. The most prominent fragments and their relative intensities are listed in table 6.

Table 6. Mass Fragments of Papaverine

m/e	Relative Intensity %		m/e	Relative Intensity %
340	12.9	M <sup>+1</sup>	280	10.8
339	69.3	M <sup>+</sup>	278	10.1
338	95.2	M <sup>-1</sup>	266	10.0
324	100.0	(base)	264	10.9
322	15.2		154	13.7
309	12.5	Other mass spectrum data have been reported (24-25).		
308	29.0			
294	13.4			
293	19.5			
292	13.2			



**FIG. 8 : THE MASS SPECTRUM OF PAPAVERINE HCL.**

### 3. Isolation of Papaverine

*Papaverine can be either prepared by isolation from opium (which contains about 1% papaverine) or by total synthesis of the alkaloid.*

The alkaloid is precipitated from the mixed hydrochlorides of opium alkaloids by sodium hydroxide solution or by sodium acetate solution together with noscapine and thebaine (26). This mixture is dissolved in dilute alcohol, acidified with acetic acid and treated with boiling water, upon which papaverine and noscapine are precipitated and separated by filtration.

Separation of these two alkaloids can be effective by the formation of their acid oxalate salts. Papaverine acid oxalate crystallizes from the mother liquor, while noscapine acid oxalate remains soluble in the solution (27,28). Papaverine is then liberated and purified by crystallization from alcohol (29).

*The isolation scheme of papaverine is presented in Fig. 9.*

A chromatographic method for the isolation of papaverine has been adopted for biosynthetic studies (30).

The acidic aqueous solution containing the total alkaloids of opium is extracted several times with chloroform. The combined chloroform extracts are evaporated to dryness and the residue is dissolved in a minimum amount of chloroform, applied into chromatographic columns of silica gel and eluted with a mixture of chloroform-ethanol (98:2). The fractions containing papaverine are combined and evaporated to dryness. Further purification can be effected by dissolving the residue in a minimum amount of chloroform and applied to silica gel preparative TLC and eluted with the solvent benzene-ethanol (4:1). The bands containing papaverine are scraped off and extracted with warm methanol. Evaporation of methanol and crystallization from ether affords crystals of papaverine.

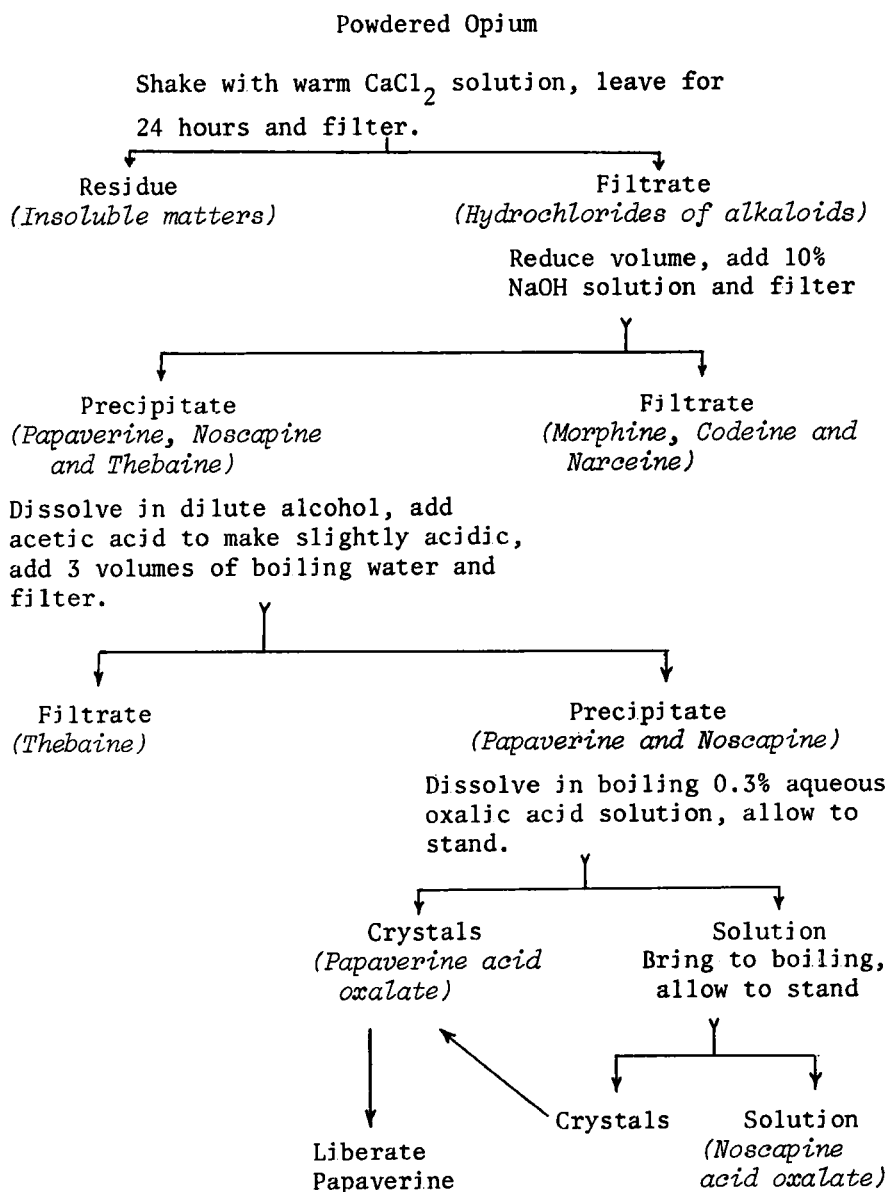


Fig. 9: Isolation Scheme of Papaverine from Opium

#### 4. Synthesis of Papaverine

The synthesis of papaverine was first accomplished in 1909 by Pictet and Gams ( 3 ), by a method which confirmed the accepted formula. The method is as follows:- Veratrole [I] is converted by Friedel-Crafts reaction into acetoveratrone [II]. This upon treatment with methyl-nitrite in HCl affords the isonitroso-derivative [III] which is reduced with tin and HCl to aminoacetoveratrone [IV]. Interaction of [IV] with homoveratroyl chloride [V] yields the amide [VI]. This by selective reduction with sodium amalgam gives homoveratroylhydroxy-homoveratrylamine [VII]. Upon heating [VII] in xylene and phosphorous pentoxide, two molecules of water are lost and the iso-quinoline ring is closed to give papaverine [VIII]. *The first synthesis of papaverine is shown in scheme I.*

The above synthesis has since been simplified and improved as follows ( 4 ):- Veratrole [I] readily undergoes chloromethylation with formaldehyde and hydrochloric acid to give the chloro-compound [II] which gives with potassium cyanide 3,4-dimethoxybenzylcyanide [III], from which homoveratrylamine [IV] and homoveratric acid [V] are produced by catalytic reduction (Raney nickel) and hydrolysis respectively ( 31 ). [IV] and [V] are condensed to give the adduct [VI] which when heated with phosphorous pentoxide in xylene affords dihydropapaverine [VII]. This undergoes dehydrogenation with palladised asbestos at 200° to yields papaverine [VIII].

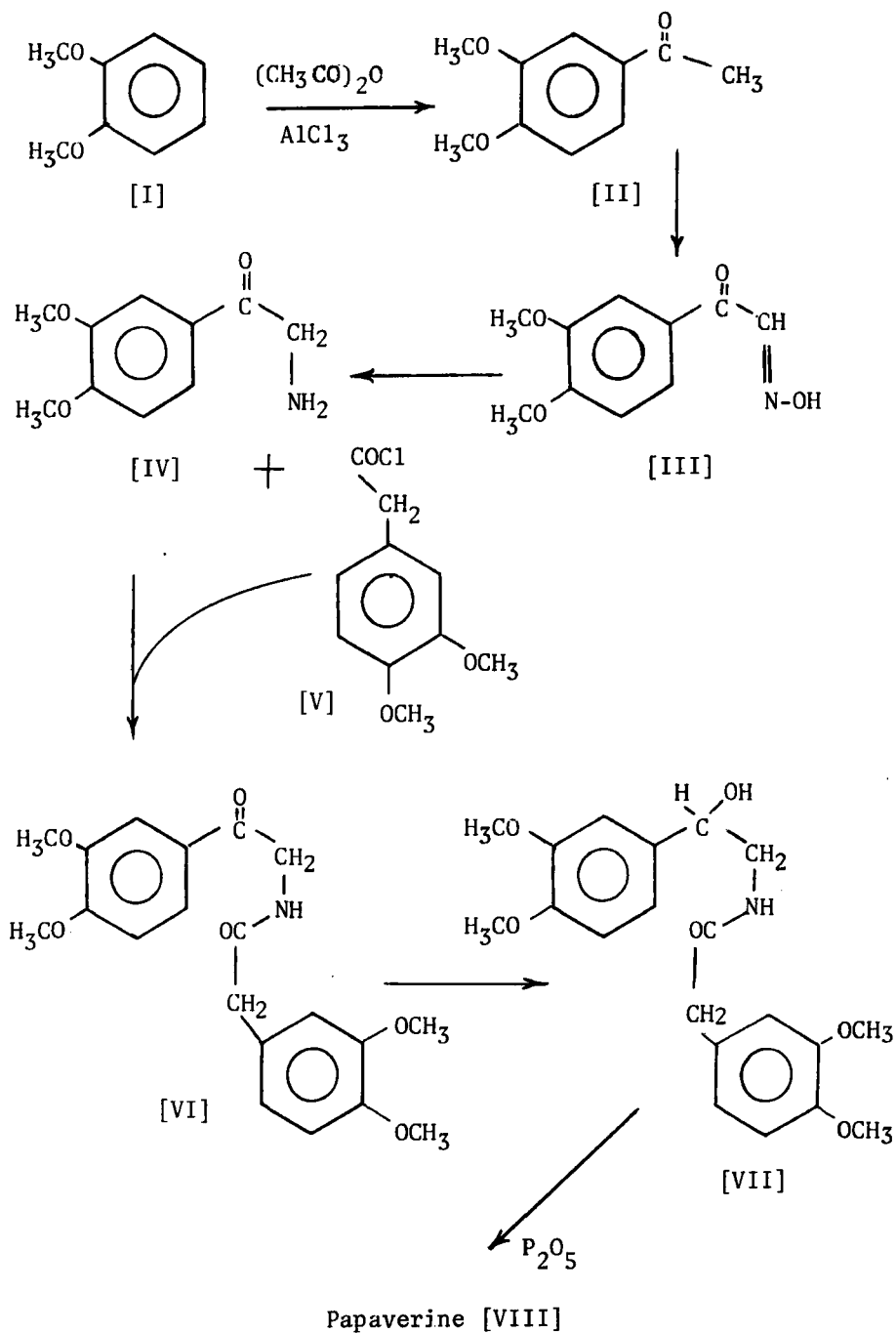
*This improved synthesis is presented in scheme II.*

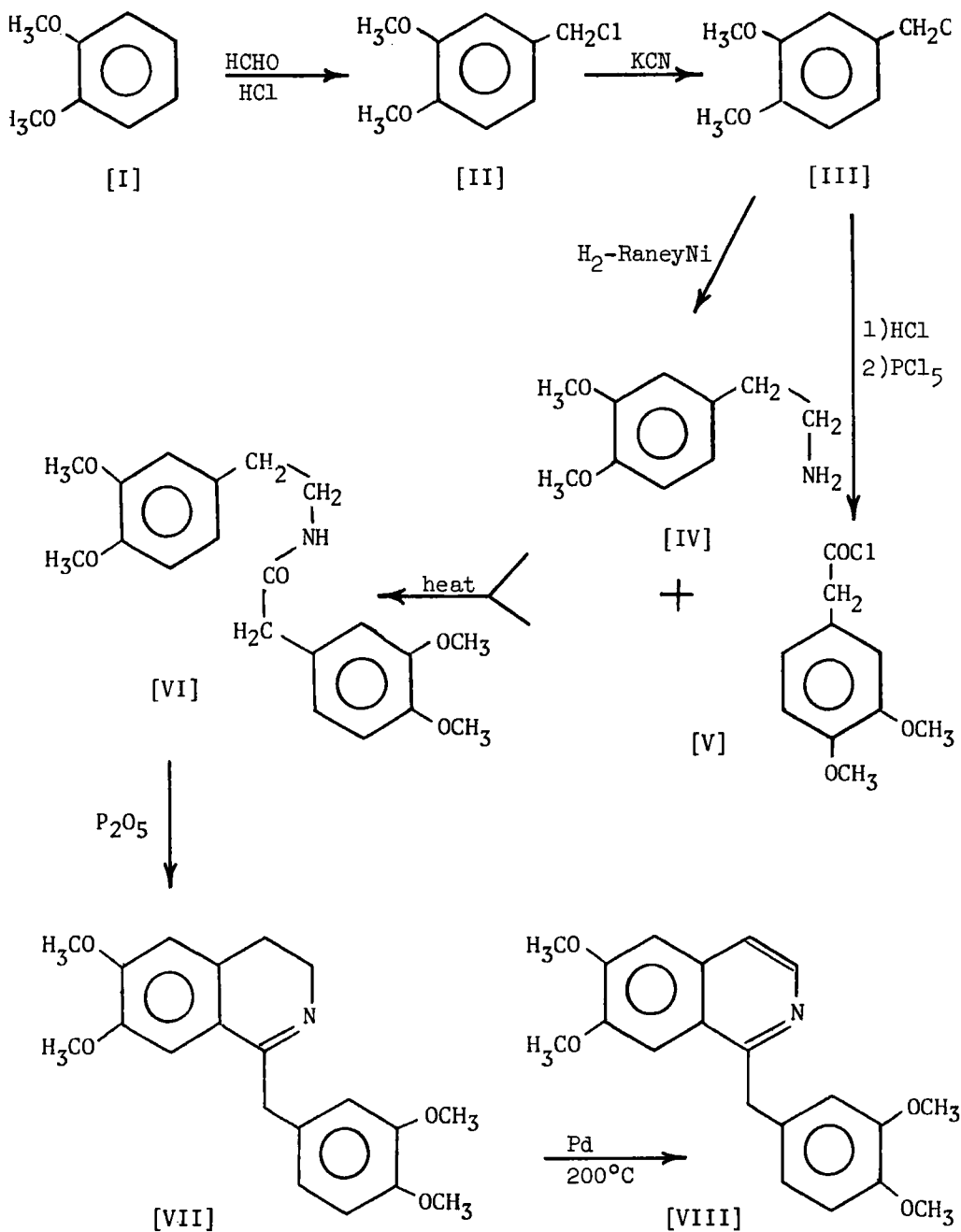
Further improvements were also achieved in two almost identical syntheses (6,7). In these, 1-methoxy-1-(3,4-dimethoxyphenyl)-2-nitroethane [II] is prepared from 3,4-dimethoxy- $\beta$ -nitrostyrene [I]. The nitro group of [II] is then reduced to give the amino derivative [III] which is condensed with homoveratroyl chloride [IV] to produce the adduct [V]. This is cyclized with phosphorus oxychloride when it loses both water and methanol and yields papaverine directly [VI].

*These syntheses are presented in scheme III.*

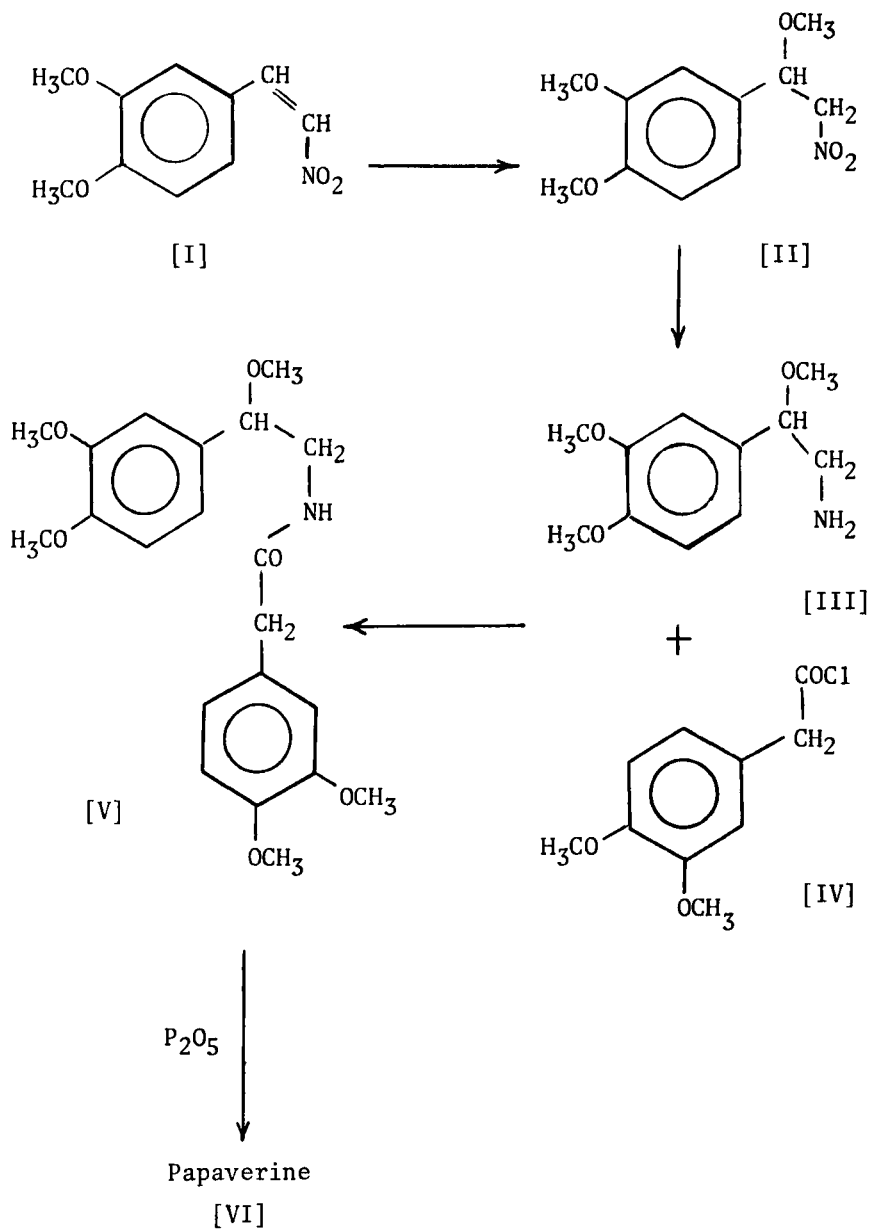
A rather different approach was carried out as follows (5) :

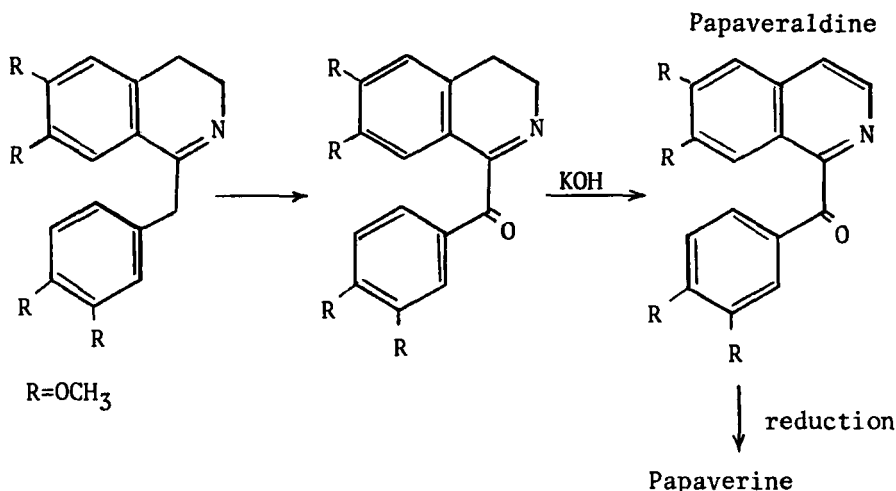
Air oxidation of 3,4-dihydropapaverine yields 3,4-dihydropapaveraldine which may be dehydrogenated by the action of potassium hydroxide, since papaveraldine is reduced to papaverine, this constitutes another synthesis of this alkaloid.

*Scheme I : First Synthesis of Papaverine*

*Scheme II : Synthesis of Papaverine*



*Scheme III : Improved Synthesis of Papaverine*



A remarkable improvements have also been reported by Kindler and Peschke (8). The authors improved a number of intermediate steps, such as the preparation of homoveratric acid from acetoveratrone by a Willgerodt reaction, formation of the amide by heating the acid and homoveratrylamine in tetralin solution and dehydrogenation of 3,4-dihydropapaverine by heating the compound with palladium black in a hydrogen-acceptor medium such as dihydrophellandrene, where good yield of papaverine were obtained.

This method formed the basis of industrial syntheses of papaverine (12).

A second commercial synthesis of papaverine was performed later by Wahl (9a) and then by Galat (9b).

Synthesis III has also been modified by Teitel and Brossi (32). The nitrostyrene [1] adds methanol under basic conditions to yield [2] this is reduced with 2.7% Na/Hg in 50% acetic acid to the aminoderivative [3]. This is condensed with homoveratroyl chloride to give the amide [4], which on Bischler-Napieralski cyclization with phosphorous pentoxide in toluene affords papaverine [5].

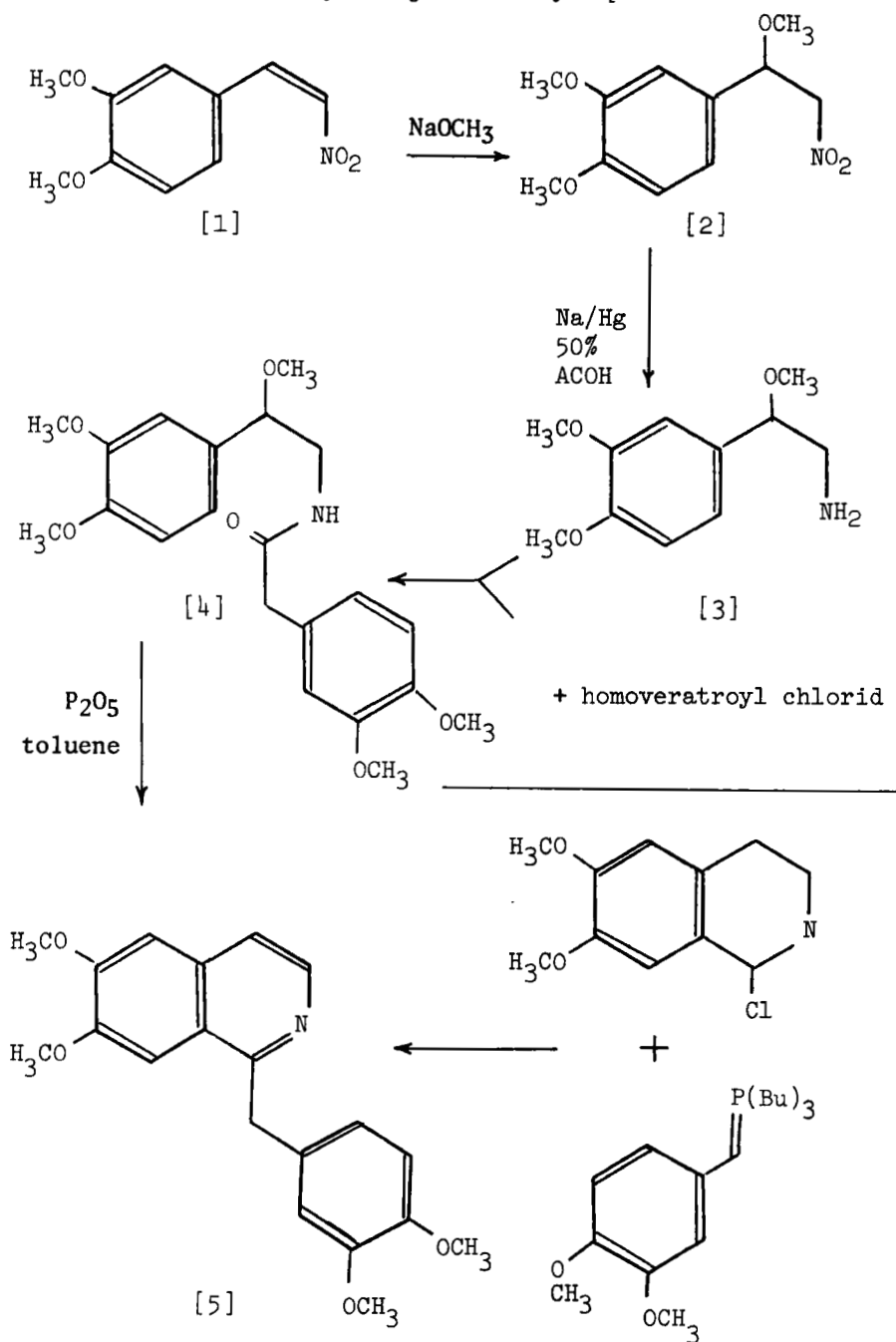
This modification is presented in scheme IV.

*All the above synthetic methods depended on the Bischler-Napieralski cyclization reaction which is also known as isoquinoline ring closure (33).*

Papaverine continues to stand as an important drug in medicine, accordingly many new synthetic methods have been elaborated (34-37) as well as many modifications of the above methods have been reported (38-41).

Sagasawa et al. (34) reported a one-step synthesis of papaverine by condensing  $\beta$ -methoxy-3,4-dimethoxyphenylethylamine with homoveratric acid.

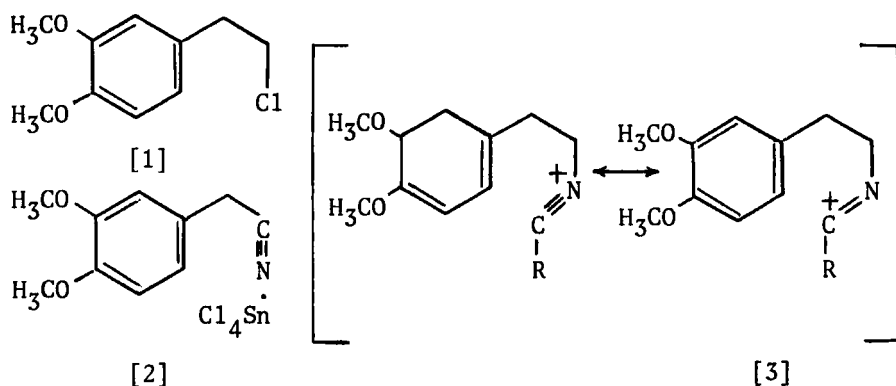
## Scheme IV : Modified Synthesis of Papaverine



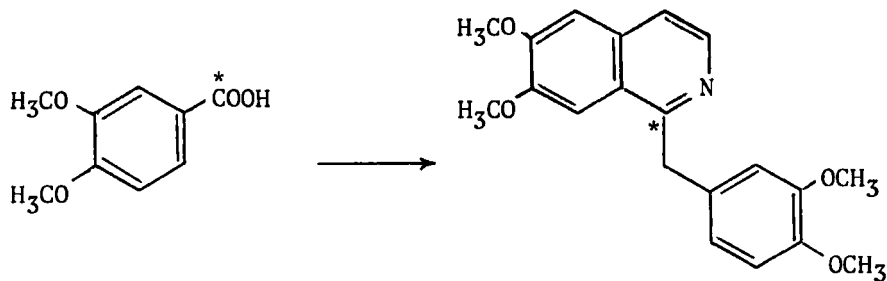
Taylor-Martin synthesis

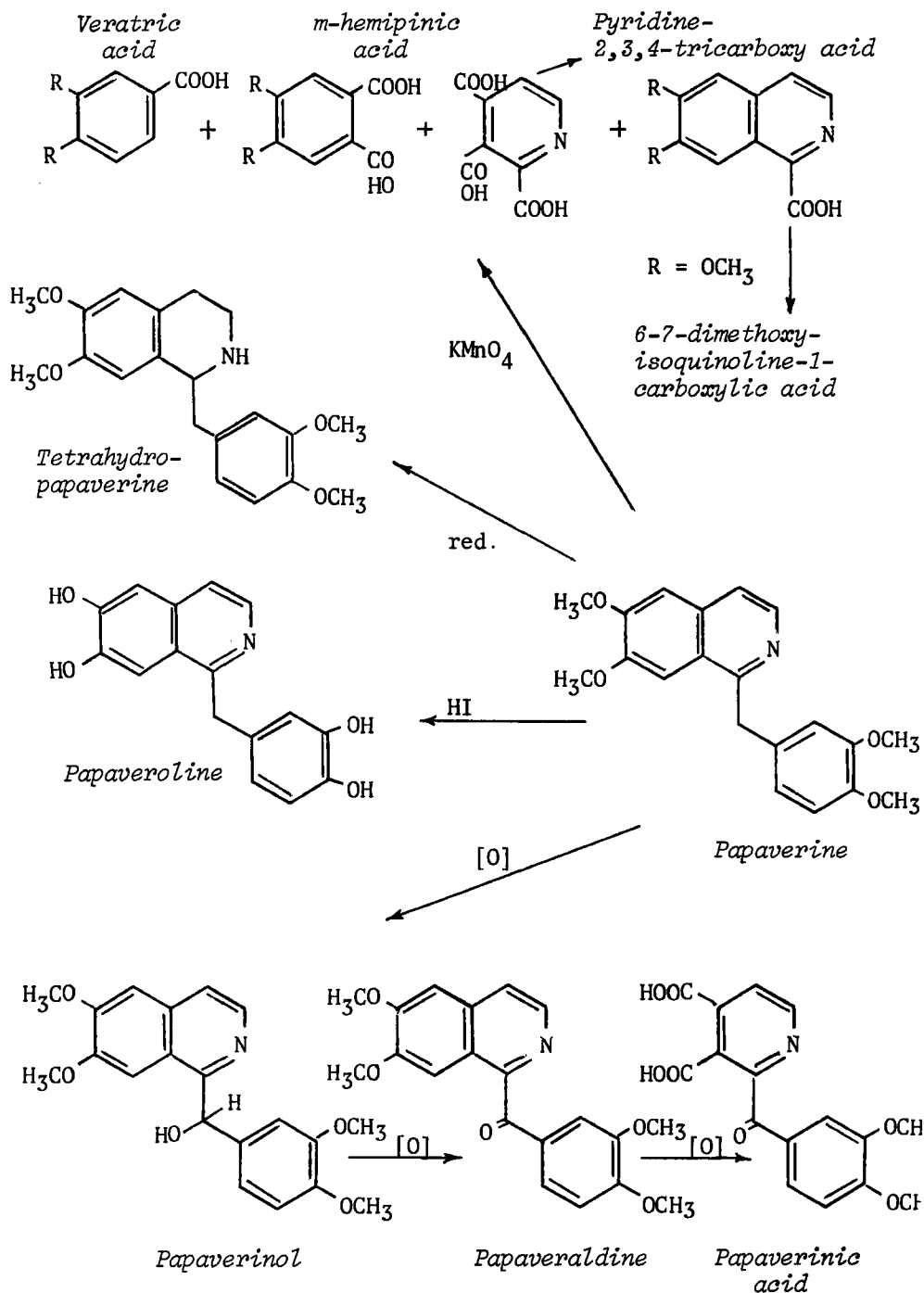
Taylor-Martin ( 35 ) also have reported another one-step synthesis of the alkaloid, involving the reaction of the Wittig reagent with 6,7-dimethoxy-1-chloroisoquinoline (which is presented in scheme IV).

3,4-Dihydropapaverine was obtained as picrate, by condensing  $\alpha$ -chloro- $\beta$ -(3,4-dimethoxyphenyl)-ethane [1] with homoveratronic nitrile-stannic chloride [2], a reaction which involves the ring closure of the mesomeric ion [3], 3,4-dihydropapaverine so produced is then converted into papaverine (36).



Labeled [ $1-^{14}\text{C}$ ] papaverine has been synthesized by the usual method of synthesis and using veratric acid labeled at the carboxylic group as starting material (37).





Some Reactions of Papaverine (10,12).

## 5. Biosynthesis of Papaverine

It has been suggested by Winterstein and Trier ( 42 ) that the benzyliisoquinoline alkaloids are derived from two units of 3,4-dihydroxyphenylalanine (dopa), one of which is transformed into 3,4-dihydroxyphenylethylamine [II] by decarboxylation, and the second into 3,4-dihydroxyphenylpyruvic acid [III] by oxidative deamination. Biological condensation of [II] and [III] in a similar manner to Pictet-Spengler condensation(43) would then afford norlaudanosoline [VI] probably through norlaudanosoline carboxylic acid [IV] and 1,2-dehydro norlaudanosoline [V]. Buttersby et al.(44-46) have tested the above theory and shown that papaverine is biosynthesized in the opium poppy from tyrosine [I] through dopa and norlaudanosoline [VI].

Brochmann-Hassen et al. ( 47 ) established that nor-reticuline is an efficient precursor of papaverine, upon the administration of radioactive ( $\pm$ )-nor-reticuline (once 3- $^{14}\text{C}$  and another 6-O- $^{14}\text{C}$  H $_3$ ) to *Papaver somniferum* plants, resulted in the isolation of labeled papaverine. The late stages in papaverine synthesis (O-methylation and aromatization) have been established by Brochmann-Hanssen et al. ( 48 ) who showed that (-)-norlaudanosoline [VI] was incorporated into papaverine via (-)-nor-reticuline [VII] or (-)-nororientaline [VIIa] to (-)-norlaudanine [VIII] or (-)-norcodamine [VIIIa] to (-)-tetrahydropapaverine [IX] and thence to papaverine [X] (Fig.10).

Kapil and co-workers ( 49 ) almost reached to similar conclusions.

*The biosynthesis of papaverine is presented in scheme V.*

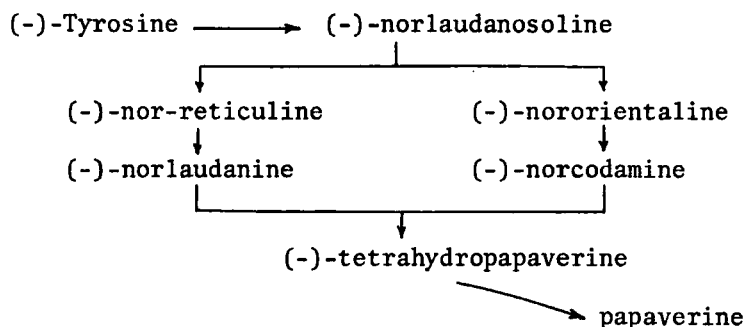
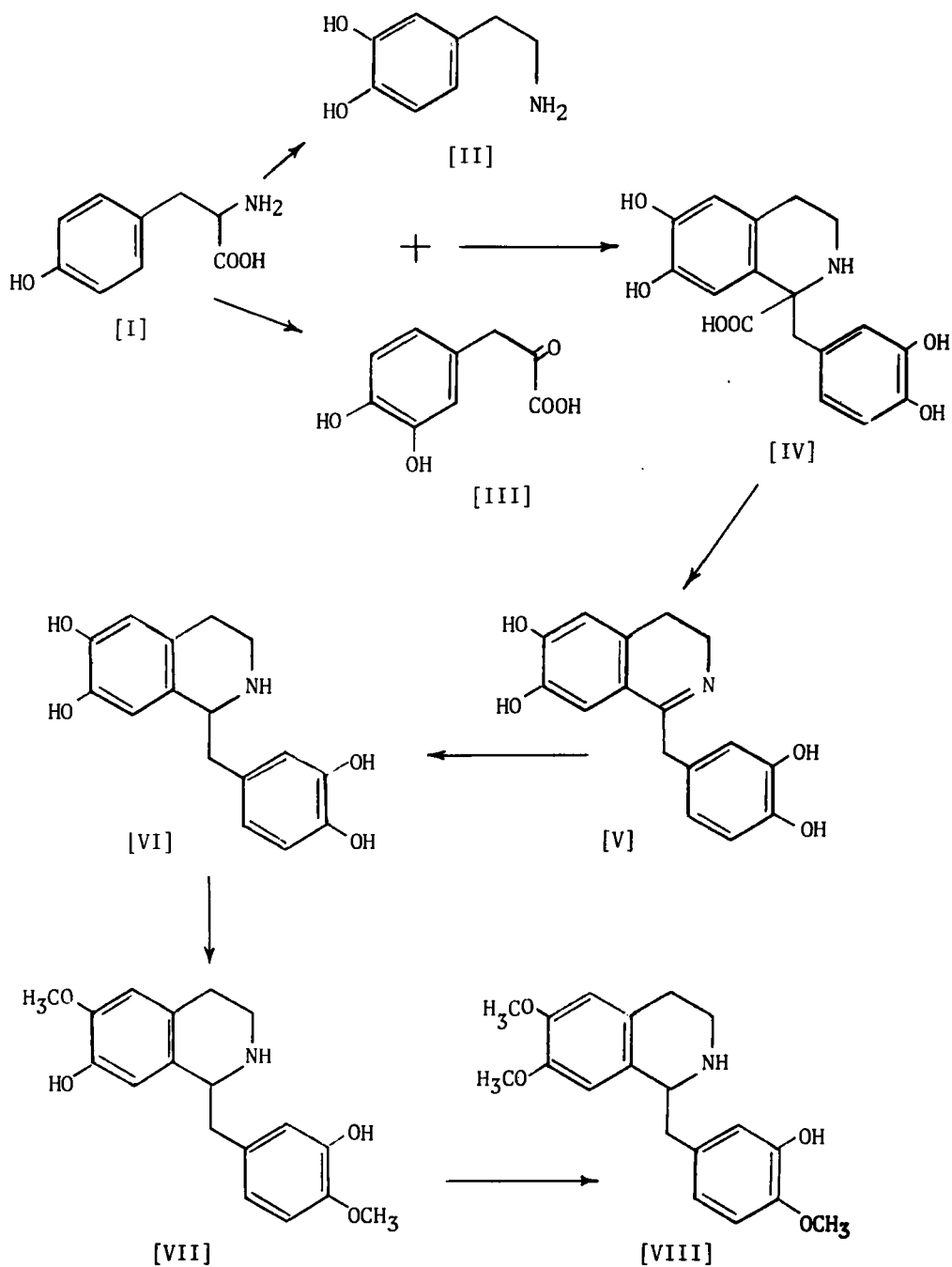
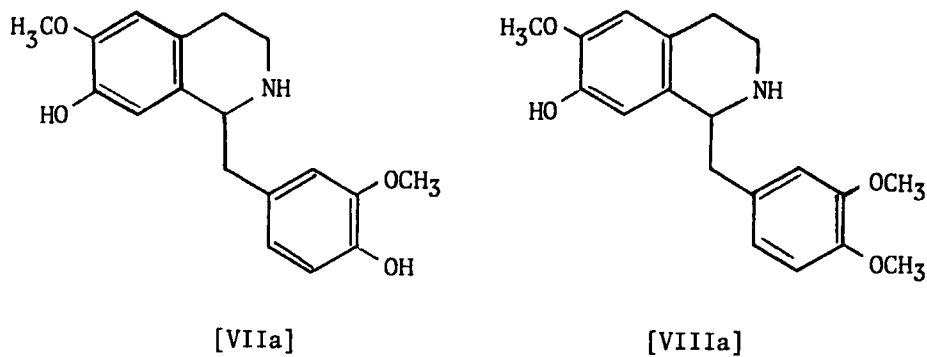
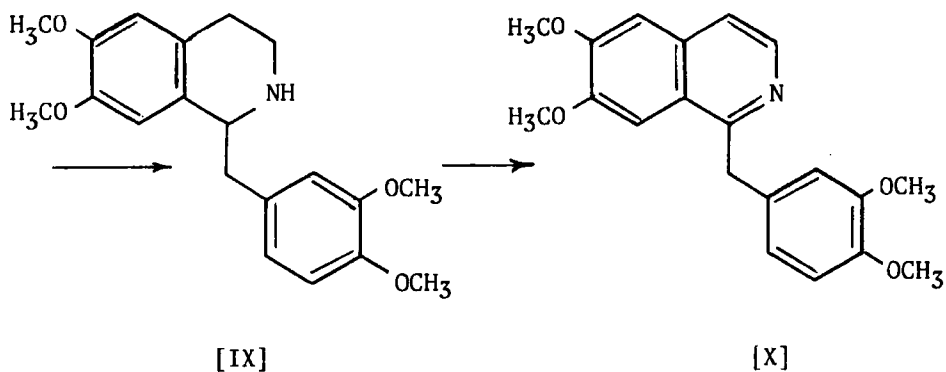


Fig. 10 : Pathway for Biosynthesis of Papaverine (48).

*Scheme V : The Biosynthesis of Papaverine*





## 6. Drug Metabolism and Pharmacokinetics

Papaverine is readily and completely absorbed after oral administration and rapidly metabolised (20, 50-52). Blood concentration : After an intravenous dose of 3 mg/kg of the hydrochloride, plasma concentrations of about 1.9 µg/ml are attained in 30 minutes and 0.6 µg/ml in 2 hours; after an oral dose of 3 mg/kg of the hydrochloride, a peak plasma concentration of 0.6 µg/ml is attained in 1 to 2 hours (50,51,53). After a single oral dose of 200 mg of [<sup>14</sup>C]-papaverine HCl, a peak concentration of 503±108 ng appeared in 2 hours and it corresponded to 8.3±2.4% of the total plasma radioactivity at this time (52).

Bioavailability : Oral bioavailability is 54% (54). Bioavailability of sustained-release preparations is less than that of compressed tablets or elixir (53).

A recent study from the University of Florida compared the intravenous administration of 100 mg or 150 mg papaverine hydrochloride, oral administration of 100 mg and 200 mg papaverine hydrochloride in a solution form, and oral administration of 150 mg and 300 mg papaverine hydrochloride as sustained-release product A (USV, 52-153) and sustained-release product B (USV, 52-162); they observed a 70% first pass metabolism for the oral solutions, independent of dose and absorption rate. Product A had an absolute availability from a sustained-release form of 22%, and product B had 29%. From an oral solution, the availability was 68% for A and 89% for B. The average peak plasma papaverine concentration from 150 mg of sustained-release A was 0.201 µg/ml, and from B was 0.271 µg/ml. Eight hours after dosing, the plasma papaverine concentrations were less than 0.05 µg/ml in all subjects (55).

Half-life : The following values have been reported: Plasma half-life, about 100 minutes (50); about 7 hours (20); during the first 6 hours about 1 hour, followed by slower elimination (52). Half-life of papaverine following administration of 150 mg of elixir or capsules was 1.2 hours (56); it was 0.5-2 hours (53).

Distribution : In the dog, papaverine is localized in the fat and liver and uniformly distributed in other tissues; protein binding, in the dog 66 to 87% bound to plasma protein (50,51).

Metabolites : The drug is mainly metabolised in the liver and excreted in the urine almost entirely as metabolites (57). It is metabolised by demethylation and glucuronic acid and sulfate conjugation of the resulting phenolic groups (20,50).

About 50-80% of a dose is excreted in the urine in 48 hours as conjugated phenolic metabolites, mostly 6-hydroxy-papaverine (37% of the dose) and 4'-hydroxypapaverine (about 8.5% of the dose). Less than 1% of a dose is excreted unchanged in the urine (20). When [ $^{14}\text{C}$ ]-papaverine was administered to human beings, a total of six radioactive metabolites were identified by TLC (52). Two of these were identified as 4'-demethylated papaverine ( $16.3 \pm 0.6\%$ ), 3'-demethylated papaverine ( $1.8 \pm 1.1\%$ ) and four unidentified metabolites as metabolite III ( $0.9 \pm 0.1\%$ ), metabolite IV ( $23.3 \pm 0.5\%$ ), metabolite V ( $9.2 \pm 1.2\%$ ) and metabolite VI ( $0.19 \pm 0.1$ ) as well as unchanged papaverine ( $0.6 \pm 0.1\%$ ).

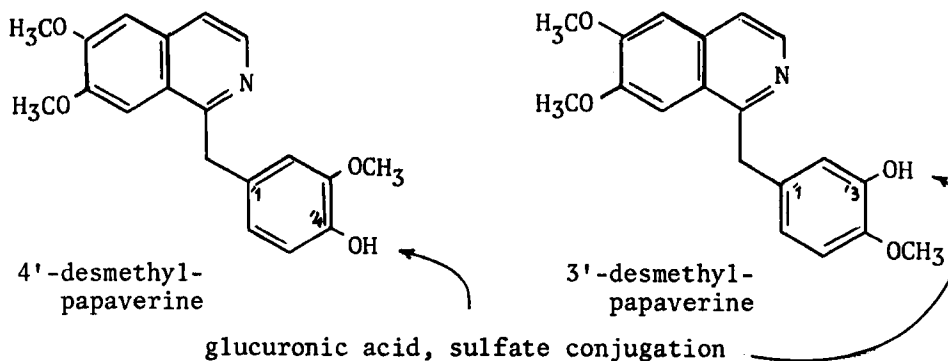
## 7. Drug Stability

Papaverine hydrochloride should be stored in airtight containers, protected from light (50). Papaverine hydrochloride preparations should be stored at a temperature less than  $40^\circ$ , preferably between  $15-30^\circ$ , freezing the injections should be avoided (58).

Papaverine hydrochloride injections which had changed from colorless to yellow on storage were found by paper chromatography to contain papaveraldine, papaverinol and 8 unidentified compounds (59).

Papaverine hydrochloride solutions, sterillised by autoclaving or filtration, developed a pale yellow color on storage for a year at  $25^\circ$ , or a month at  $37^\circ$ , but there was no loss of potency after 4 years at  $25^\circ$  or 18 months at  $37^\circ$ . The addition of 0.005% sodium edetate inhibited color formation at pH 3 to 4 for at least 2 years, probably by the chelation of iron. Light and air increased discoloration (57,60).

Papaverine hydrochloride injection, should not be added to lactated Ringer's injection because a precipitate would result (58).



## 8. Methods of Analysis

### 8.1 Identification Tests

The following identification tests are mentioned under papaverine hydrochloride in the British Pharmacopoeia (15) and its 1982 Addendum (14):-

To 10 mg of papaverine hydrochloride add 3 ml of acetic anhydride, cautiously add 0.15 ml of sulfuric acid and heat on a water bath for 3 to 4 minutes; a yellow color with green fluorescence is produced.

To 10 ml of a 2% w/v solution add dropwise 10 M ammonia and allow to stand. Melting point of the precipitate, after washing with water and drying, 146-149°.

The light absorption, in the range 230 to 270 nm, of a 0.0005 % w/v solution in 0.01 M hydrochloric acid exhibits a maximum only at 250 nm; specific absorbance at 250 nm, 1590 to 1670. The light absorption in the range 270 to 350 nm, of a 0.0025% w/v solution in 0.01 M hydrochloric acid exhibits two maxima at 280 to 290 nm, and 303 to 313 nm; specific absorbance at these maxima, 140 to 200 and 200 to 250 respectively.

Yields the reactions characteristic of alkaloids and reaction characteristic of chlorides.

Dissolve a few mg of papaverine HCl in 5 ml of water, add 1 ml of potassium iodobismuthate solution, an orange or orange-red precipitate is produced.

The following tests are mentioned in the USP (61):

The infrared absorption spectrum of a potassium bromide dispersion of it, previously dried, exhibits maxima only at the same wave lengths as that of a similar preparation of USP Papaverine Hydrochloride RS.

The ultraviolet absorption of a 1 in 400,000 solution in 0.1 N hydrochloric acid exhibits maxima and minima at the same wavelengths as that of a similar solution of USP Papaverine Hydrochloride RS, concomitantly measured and the respective absorptivities calculated on the dried basis, at the wavelength of maximum absorbance at about 251 nm do not differ by more than 3.0%.

A solution (1 in 50) responds to the tests for chloride.

The following tests are used to identify papaverine

#### Warren's Test

Mix a crystal of potassium permanganate and 0.5 mg papaverine with a glass rod, add 0.2 ml of Marqui's reagent with stirring; a green color appears which rapidly changes to blue.

Ammonium molybdate test gives blue or faint green color with papaverine (19) (sensitivity: 0.5  $\mu$ g).

Ammonium vanadate test gives grey green color (19) (sensitivity: 1.0  $\mu$ g).

Vitali's Test gives faint yellow/orange/brown (19).

Pure papaverine gives no color reaction with cold concentrated sulfuric acid (62).

### 8.2 Microcrystal Formation

The microcrystals of papaverine were performed on a solution of papaverine hydrochloride in water (1 mg in 1 ml). 1 to 2 drops of this solution were treated with an equal quantities of the specific reagent on a microscopical slide. After a specific time, the crystals so formed were microscopically examined. Shapes of the crystals are presented in table 7 (63).

Table 7 : Microcrystal Formation of Papaverine

Plate	Reagent	Time of formation minutes	Shape of crystals
1	Zinc chloride (5% aq. solution)	7	Transparent rectangular plates (sensitivity: 1 in 3000) (19).
2	Mercuric chloride (5% aq. solution)	4	Radiating rods mostly in 2 to 3 per crystal.
3	Picric acid (saturated solution)	6	Large prisms.
4	Platinic chloride	5	Small irregular rectangles.
5	Potassium-cadmium iodide (Marm's)	10-15	Small stars gradually changing to radiating rods.
6	Wagner's	4	Rosettes with irregular sizes.
7	Potassium chromate solution	10	Radiating needles.
8	Sodium carbonate	5	Long radiating needles.

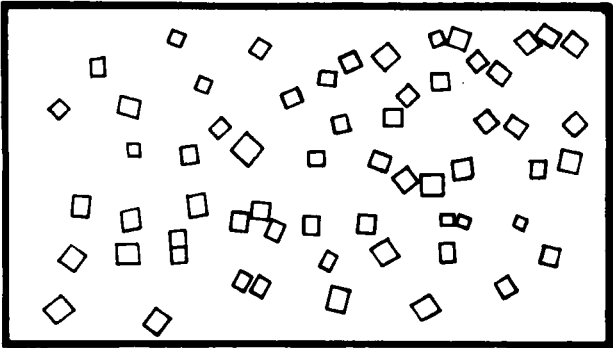


PLATE 1. MICROCRYSTALS OF PAPAVERINE WITH ZINC CHLORIDE

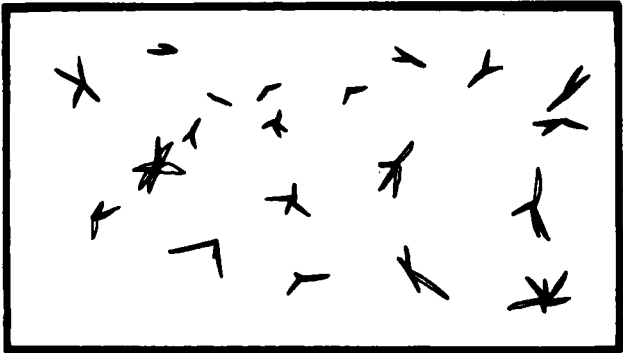


PLATE 2. MICROCRYSTALS OF PAPAVERINE WITH MERCURIC CHLORIDE

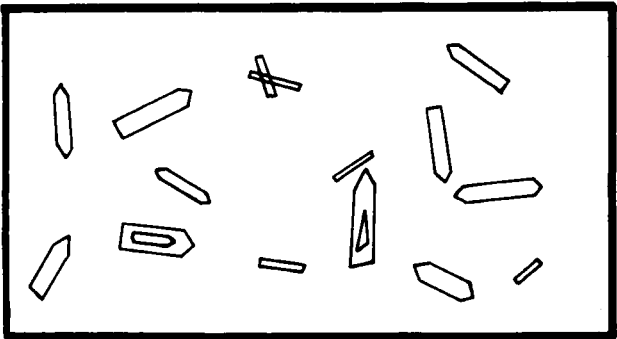


PLATE 3. MICROCRYSTALS OF PAPAVERINE WITH PICRIC ACID



PLATE 4. MICROCRYSTALS OF PAPAVERINE WITH PLANTINIC CHLORIDE

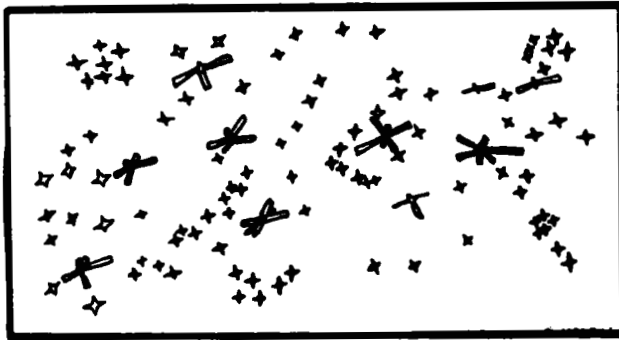


PLATE 5. MICROCRYSTALS OF PAPAVERINE WITH MARM'S REAGENT

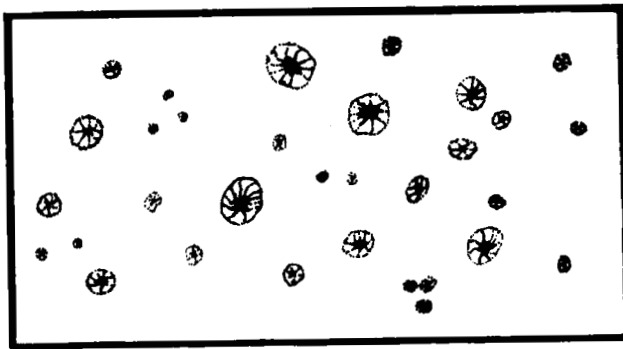


PLATE 6. MICROCRYSTALS OF PAPAVERINE WITH WAGNER'S REAGENT

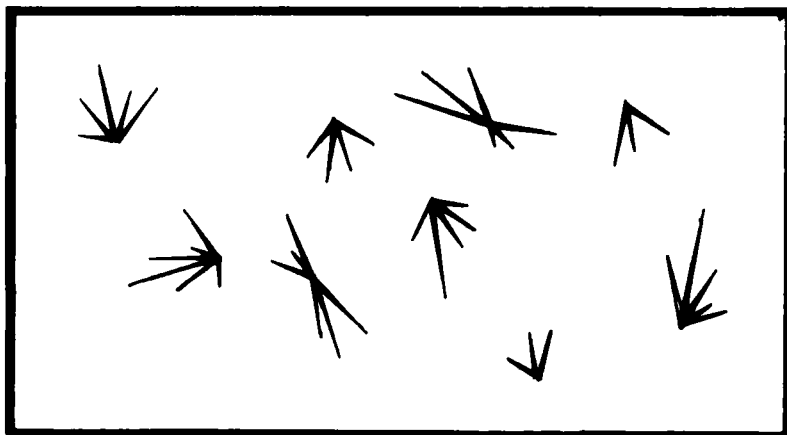


PLATE 7. MICROCRYSTALS OF PAPAVERINE WITH POTASSIUM CHROMATE

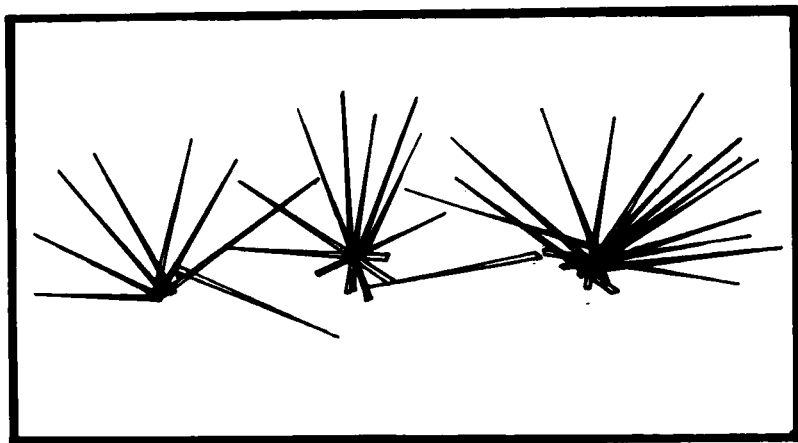


PLATE 8. MICROCRYSTALS OF PAPAVERINE WITH SODIUM CARBONATE

### 8.3 Titrimetric Methods

#### 8.3.1 Non Aqueous Titration

The non-aqueous methods for determination of papaverine in pharmaceutical preparations was adopted by certain pharmacopoeias (European Pharmacopoeia 1969, Egyptian Pharmacopoeia 1972, B.P. (1980) and USP XX (64-66,61).

The method was carried out as follows:

Dissolve 0.3 to 0.4 g accurately weighed amount of papaverine-HCl in 30 ml glacial acetic acid. Titrate against 0.1 M perchloric acid using 6 to 7 ml of mercury (II) acetate solution and using crystal violet solution as indicator.

Each 1 ml of 0.1 M perchloric acid equivalent to 0.03759 g of papaverine-HCl.

A method was given for the determination of various alkaloids including papaverine, by titration in non aqueous media with 0.1 N of the HCl complex of chloroaluminium isopropoxide which behaves as a monobasic acid when undergoing salt formation with various alkaloids. The deviation was  $\pm 1\%$  in the range 38-245 mg of alkaloid (67).

Arylsulfonic acids in 0.005 N dioxane solutions were used for quantitative determination of different alkaloids including papaverine (68). The titrants contained 1% PhOH and were standardized against atropine or brucine dissolved in  $\text{CHCl}_3$  using 0.1% dimethyl yellow as indicator. For the determination, 5 ml of a solution to be analyzed were taken containing 5 mg alkaloid salt, its pH adjusted to 8-9 with saturated  $\text{NaHCO}_3$  solution or 5% NaOH, and the solution was extracted, 4-5 times with 5-10 ml  $\text{CHCl}_3$  each. The combined extract were filtered and titrated. Error of determination was  $< 1\%$ .

Other non aqueous method, for the determination of papaverine were also discussed (69)

#### 8.3.2 Selective Chromatographic Determination

$\text{CHCl}_3$  or  $\text{Et}_2\text{O}$  solutions of papaverine was passed through 15-20 cm, tall and 10-12 mm diam. columns of tartaric or citric acid at 4-5 drops/sec. The column packing was dried at  $50-60^\circ$



and eluted with  $\text{CHCl}_3$ . Papaverine was eluted as free base and can be determined by the conventional titrimetric method (70).

Other volumetric titration methods for papaverine assay in plant extracts or pharmaceutical preparations were also mentioned (71,72).

### 8.3.3 Complexometric Titration

Complexometric titration with Pb (II) picrate was applied for determining papaverine-HCl in multicomponent preparations; the std. deviation did not exceed  $\pm 0.5 \times 10^{-3}$  g (73).

### 8.3.4 Heterometric Titration

A microheterometric titration of papaverine using tungstosilicic (I), tungstophosphoric (II) or molybdophosphoric acids (III) at pH 1 or 7 was published (74): Titrate 20 ml of 0.001-0.002 M soln. of the alkaloid, heterometrically with 0.00125-0.005 M of I, II or III. The molar ratios of the stoichiometric compound formed in pH 1 (HCl) or pH 7 (NaOAc) solution are papaverine/I 2, - (no ppt.), /II 3/2. The recoveries were quantitative. Micro amounts of the reagents I, II and III were quantitatively determined by a reverse heterometric titration with the alkaloid: titrate 20 ml of 0.0085-0.0005 M of I, II or III containing 1 ml of M HCl (or 2 ml of M NaOAc) heterometrically with 0.01-0.00025 M of papaverine.

Another method which silico-tungstic acid was used as a titrant for determination of papaverine - HCl was reported (75).

## 8.4 Electrochemical Methods

### 8.4.1 Polarometric Titration

A polarometric titration procedure for the determination of papaverine-HCl was discussed. The dropping Hg electrode was used as cathode and saturated calomel electrode as anode. Phosphotungstic acid and picric acid solutions and alizarin sulfonate solution were used for the assay (76).

Polarographic reduction of papaverine and 3,4-dihydropapaverine in aqueous methylammonium hydroxide gave half-wave potential of - 1.92 V and - 1.52 respectively (77).

#### 8.4.2 Potentiometric Titration

Papaverine-HCl was potentiometrically titrated with 2.5% Na tetraphenyl borate using the valinomycin ion selective electrode (78 ).

Another potentiometric method (79) based on the formation of insoluble alkaloid picrate salts, using a picrate ion-selective indicating electrode was described for the determination of papaverine-HCl. The method was successfully applied to pharmaceutical preparations

#### 8.5 Radiometric Titration

A radiometric titration method has been developed to enable micro determination of different alkaloid salts including papaverine-HCl. For hydrochlorides, add a known volume of concentrated  $\text{HNO}_3$  to 0.5-3 ml of alkaloid hydrochloride in a centrifuge test tube, to result in 0.7-0.8 M  $\text{HNO}_3$ . Then titrate with 0.01 M  $\text{AgNO}_3$ . After each addition of  $\text{AgNO}_3$  solution, agitate to homogenize and centrifuge with complete sedimentation of the ppt., transfer a known volume of the clear solution to the  $\gamma$ -counter, and after counting, return to the test tube, and repeat sequence. It is necessary to make at least 3 additions of titrating agent before the equivalent point and at least 3 additions after it. The equivalent point was determined graphically by extrapolation, with an error of  $\pm 0.4\%$ . This method can be adapted to mixtures of the alkaloid salts by titrating at different pH values (80).

Another radiometric titration method for papaverine, with potassium thallium iodide reagent, was reported (81 ). The sample solution containing 0.01 N acid was reacted with excess titrant within a total solution volume of 5 ml. The ppt. of (alkaloid H)  $\text{TlI}_4$  was separated by centrifuging and the  $\beta$  activity of a supernatant aliquot was measured by using a Geiger counter. The errors were within  $\pm 2.0\%$  for determining 4-6 mg alkaloid alone and they were with  $\pm 3.2\%$  for determining 7-15 mg alkaloid in pharmaceutical preparations.

## 8.6 Gravimetric Methods

A gravimetric method for determination of papaverine and its salts was mentioned (69). The base may be extracted by chloroform or  $\text{CCl}_4$  from alkaline solution and weighed after drying at  $105^\circ$ . 1 g residue = 1.107g papaverine HCl or 1.144 g of papaverine sulfate.

Another method was proposed for gravimetric determination of papaverine in papaveretum powder (82). The method recommended was as follows: Dissolve 1 g of sample in 20 ml of water in a separator and add 40 ml of  $\text{CHCl}_3$ . Add 1 drop of dil. HCl and shake vigorously for 30-40 seconds. After separation, wash the  $\text{CHCl}_3$  first with 10 ml of N NaOH and then with 10 ml water. Repeat the extraction with 4x20 ml portions of  $\text{CHCl}_3$ , washing each extract as before with NaOH and  $\text{H}_2\text{O}$ . Filter the washed  $\text{CHCl}_3$  extracts into a dry flask, recover the  $\text{CHCl}_3$ , add 2 ml of dehydrated ethanol and evaporate on a water-bath in a current of air with constant rotation of the flask, avoiding undue heating. Dissolve the crude residue (narcotine and papaverine) in 6 ml of  $\text{C}_6\text{H}_6$ , add 3 ml of a 10% w/v solution of KOH in dehydrated ethanol, and place in a water-bath at  $20^\circ$  for 40 min. Transfer to a separator, washing in with three quantities of 5 ml of  $\text{C}_6\text{H}_6$  and then with 10 ml of N NaOH. Shake well, separate; wash the aqueous layer with 10 ml  $\text{C}_6\text{H}_6$  and transfer to a small conical flask. Wash the  $\text{C}_6\text{H}_6$  layers with two 5-ml quantities of N NaOH and finally with two 5-ml quantities of  $\text{H}_2\text{O}$ , adding all washings to the first aqueous layer. Filter the  $\text{C}_6\text{H}_6$  layers into a dry flask, washing with 5 ml of  $\text{C}_6\text{H}_6$ . Evaporate and dry, with the addition of 2 ml of ethanol and heating in a current of air, with rotation. Moisten the residue with 1 ml conc. HCl, cover and allow to stand for 15 min. Wash into a separator with 20 ml of  $\text{H}_2\text{O}$  and extract papaverine with 20,10,10 and 10 ml of  $\text{CHCl}_3$ , washing each extract with 10 ml of N NaOH and then with 10 ml of  $\text{H}_2\text{O}$ . Filter the  $\text{CHCl}_3$  extracts into a tared flask, evaporate and dry with the aid of 2 ml ethanol followed by drying at  $100^\circ$  to  $105^\circ$  for 10 min, cool and weigh as papaverine.

### 8.6.1 Gravimetric-Photometric Methods

A method depends on the use of hexachlorotellurates in the analysis and control of certain

alkaloids was used for determination of papaverine HCl (83,84). The alkaloid was dissolved in HCl and precipitated as hexachlorotellurate with  $\text{H}_2[\text{TeCl}_6]$ . The ppt. was washed with HCl, dried at  $50^\circ$  and weighed. The error of the gravimetric method was  $\leq 0.75\%$ . The alkaloid in above residue after dissolution in MeOH was determined photometrically at 420 nm. The error was  $\leq 1.6\%$ .

## 8.7 Spectrophotometric Methods

### 8.7.1 Colorimetric

A colorimetric method for determination of papaverine-HCl was developed (85). The method based on measuring the blue color of the  $\text{Co}(\text{SCN})_4$  --complex in  $\text{CHCl}_3$  solution. A second method for colorimetric determination of papaverine, using Litol red, was reported (86). A third method for spectrophotometric determination of papaverine following TLC separation from other opium alkaloids was discussed (87).

Papaverine-HCl was treated with chromazurol S in 0.1 M HCl and the colored reaction product after  $\text{CHCl}_3$  extraction was measured at 455 nm. Relative error of the determination was  $\pm 1-3\%$ . Chromazurol S reacted also with 34 other alkaloids (88).

Comparative studies on various seven sulfonic azodyes for alkaloid analysis in biological fluids was reported (89). Orange II was more sensitive than methyl orange in case of papaverine determination and tolerated greater amounts of extraneous ions (such as  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{F}^-$ ,  $\text{C}_2\text{O}_4^{2-}$ ). Another visible spectrophotometric method for the assay of papaverine was mentioned (90).

### 8.7.2 Ultraviolet (UV)

A short-wave UV radiation method for the determination of papaverine when in a mixture with morphine and ephedrine has been reported (91). The absorption of each alkaloid was measured at 253.7 nm which obtained from low pressure

mercury lamp in conjunction with luminescent substance at pH 1.9. The molecular extinction coefficient,  $\epsilon$  for papaverine is  $5.767 \times 10^4$ .

UV spectrophotometry was used to follow the decomposition of papaverine, morphine, strychnine and atropine on sterilization of their 1% aq. solution by autoclaving 20 min and 2,5,7 and 10 hr at  $120^\circ$ . Two dimensional TLC was used as a ref. check. UV spectrophotometric determination was accurate with samples heated up to 2 hr; prolonged heating resulted in the formation of ppts interfering with the determination (92)

A two-dimensional TLC/UV method for determination of papaverine when present with its oxidation products papaverinol and papaveraldine was stated (93). The three components were separated by two-dimensional TLC on silufol UV 254 by using  $\text{CHCl}_3$ -acetone-aq. 25% ammonia (75:25:1) and cyclohexane- $\text{CHCl}_3$ -diethylamine (4:5:1) as mobile phases ( $R_f$  of papaverine was 0.69 and 0.54) in the two systems respectively). The spots were detected in UV radiation, cut out and extracted with 0.1 N-HCl. The extracts were centrifuged and the absorbance was measured at 309nm for papaverine. The contents were calculated from the absorbance of standard solution. The relative error of papaverine-HCl do not exceed  $\pm 2.7\%$ .

Other UV procedures for determination of papaverine-HCl injection and Tablets were recorded (61,94)

### 8.7.3 Spectrofluorimetric

A rapid spectrofluorimetric method for the analysis of synthetic mixtures resembling papaveretum (mixture of the hydrochlorides of morphine, codeine, narcotine and papaverine) which should be applicable to trace analysis in clinical chemistry as well as to the analysis of pharmaceuticals has been described (95). Accurately weigh 40 to 50 mg of the alkaloid mixture and dissolve it in 0.1 N  $\text{H}_2\text{SO}_4$ . Transfer the solution to a 100-ml standard flask and dilute to the mark with 0.1 N  $\text{H}_2\text{SO}_4$ . For papaverine, the procedure was carried out as follows: Transfer by pipette 5 ml of the alkaloidal solution into a separating funnel and add 10 ml of pH 9 buffer.

Extract with 20, 15 and 10-ml volumes of  $\text{CHCl}_3$ , allowing the separated  $\text{CHCl}_3$  layer each time to drip slowly through a  $\text{CHCl}_3$  moistened plug of anhydrous  $\text{Na}_2\text{SO}_4$  into a standard 50-ml flask. Wash the  $\text{Na}_2\text{SO}_4$  plug with a small volume of  $\text{CHCl}_3$  and dilute to the mark. This is solution P. Prepare a blank solution, BP, simultaneously by treating a 5-ml volume of water in a manner identical with that for the alkaloid solution. Measure the fluorescence intensity of the working standard solution at 320/348 nm and then measure the fluorescence intensity of solution P, subtracting that of the blank solution, BP, at the same wavelength combination. Measure the fluorescence intensity of the working standard again and average the two standard readings to calculate the  $I_{AP}$  value of solution P. Use the calibration graph for papaverine to determine the concentration of solution P. After the concentration (in  $\mu\text{g/ml}$ ) of papaverine has been obtained the percent was calculated according to the following formula:

$$\text{papaverine (\% w/w)} = \frac{\text{concentration } (\mu\text{g ml}^{-1}) \times 100}{\text{sample weight (mg)}}$$

The same article discussed the effect of temperature on the  $I_{AP}$  values of standard alkaloid solutions whereby papaverine showed a 0.7% change in relative fluorescence intensity per  $^{\circ}\text{C}$  with a percentage error in  $I_{AP}$  value 0.05.

Other spectrofluorimetric procedure for determination of papaverine have been reported (96-100).

#### 8.7.4 Mass Spectroscopy

Opium alkaloids can be determined from their mass spectra by comparison of the parent peak of each individual alkaloid with that of a reference substance added in known amounts and by reference to a calibration graph (101,102).

At a low ionization voltage, the  $M^+$  ion can be detected clearly, but the peak is small. At higher ionization voltages, the  $M^+$  ion peak becomes higher, but many other fragments peaks overlap it.

The relationship between the intensity of the  $M^+$  ion peak and ionization voltages was

studied and appropriate ionization voltages were determined.

#### 8.7.5 Ion-Pair/spectrophotometry

A study of the reactions of papaverine and quinine with acid dyes was presented and a method for the determination of papaverine in medicinal products was proposed (103):

A solution containing  $1-4 \text{ mg ml}^{-1}$  of papaverine, prepared from an injection solution or tablet, was filtered, mixed with 1 M M-gallion (1-2 ml) and adjusted to pH 6. The ion-pair complex was extracted into butanol (10 ml), and the absorbance of the extract was measured at 582 nm.

Another ion-pair method for determination of papaverine among other alkaloids was discussed (104). The method based on the formation of an ion pair with  $\text{Co}(\text{SCN})_4^{2-}$ , and extraction of the ion pair into 1,2-dichloroethane for atomic absorption spectroscopy measurement of Co at 241.0 nm

### 8.8 Chromatographic Methods

#### 8.8.1 Paper Chromatography

Several methods for separation and identification of papaverine and/or papaverine salts by paper chromatography were reported (19,105,106). The following conditions were adopted: 1-paper: Whatman No. 1, buffered by dipping in a 5% solution of sodium dihydrogen citrate, blotting and drying at  $25^\circ\text{C}$  for 1 hr; solvent, 4.8g of citric acid in a mixture of 130 ml of  $\text{H}_2\text{O}$  and 870 ml of n. butanol. The technique was ascending and the time of run was 5 hr., papaverine  $R_f = 0.49$ . location was achieved by inspection under UV; papaverine showed a pale yellow green fluorescence. 2. paper, "M" type, treated with  $\text{EtOH-HCO NH}_2$  (1:1) and  $\text{CHCl}_3 - \text{C}_6\text{H}_6$  (2:1) as a mobile phase. Samples  $\geq 1 \text{ } \mu\text{g}$  (in aq. soln.) were used for separation to make spots 0.5 cm;  $R_f$  of papaverine-HCl was 0.91.

Another method (107) reporting the use of reverse phase paper chromatography for the analysis of papaverine was also mentioned; paper,

Whatman No. 1 or No.3 impregnated by dipping in a 10% solution of tributyrin in acetone and drying in air, solvent was buffer pH 4.58; the  $R_f$  of papaverine-HCl was 0.08 using UV and iodoplatinate for location.

#### 8.8.2 Thin-Layer Chromatography TLC

Various adsorbents, solvents and techniques have been used for the TLC separation of opium alkaloids.

Bela (108) has tested out 28 different solvents and recommends double development in TLC on silica gel G layers, using 1. benzene-methanol (90+10) and 2. chloroform-ethanol-ethyl acetate-acetone (60+20+10+10) at 20°C and with a length of run of 15 cm in each case. Papaverine showed an  $R_f$  value of 75.

The  $R_f$ -values of a number of opium alkaloids and their derivatives have been determined on various adsorbents and using various solvents also by Walidi et al (109), Teichert et al (110, 111), Schwarz et al (112,113) and Braumler et al (114). According to Ramaut (115), a good separation of all these alkaloids is possible on silica gel G layers using hexane-cyclohexane-cyclohexanol (33+33+33), + 5% diethylamine. Machata (116) has applied TLC to the detection of opium alkaloids and other medicaments in the stomach contents and bile of a human being who had been poisoned with opium tincture. Teichert et al (110,111) have found benzene-heptane-chloroform-diethylamine (60+50+30+0.03) preferable with silica gel which had been alkalinated with 0.5 N KOH; they thus achieved a good fractionation of the opium alkaloids (morphine remained at the start). The Dragendorff reagent or a ferric chloride-perchloric acid mixture were used for visualisation. Neurauer and Mothes (117) have applied TLC more intensively to control of opium, especially in the selection of different types of poppy (118). They were able to separate 10 alkaloids from one another, using benzene-methanol (80+20) on silica gel layers. These authors employed TLC also for semi-quantitative determination; in this, the spots were visualised with Dragendorff reagent, photographed and the



black spots of the positive evaluated photometrically. Neurauer (119) has made use of this method in the quantitative determination of alkaloids during the growth of Papaver somniferum. Ikram et al (120) describe the separation of morphine, codeine, papaverine and thebaine on a loose alumina layer (Merck), using three solvents. Papaverine and narcotine cannot always be separated satisfactorily and this has led to the use of two-dimensional TLC (121,122). With xylene-butanone-methanol-diethylamine (40+60+6+2) in a one-dimensional procedure on activated silica gel G, Bayer (123) has succeeded in separating the following alkaloids : narcotine h R<sub>f</sub> 74, papaverine 59, thebaine 45, codeine 26, morphine 12. The problem of the papaverine-narcotine separation has thus been solved. Stahl and Jork (124) have critically compared the numerous solvents already quoted in the literature to identify crude opium and opium preparations (Fig.11). The standard method was used on silica gel G F254 layers, with uniform external conditions. The fluorescence-quenching zones of the six best known opium alkaloids could be visualized with concentrated H<sub>2</sub>SO<sub>4</sub>, which yielded differentiating colours, 10 µg amounts on heating 10 min at 150° gave grey colour with papaverine. Papaverine and narcotine can be distinguished with neutral solvents which contain benzene as chief component (Fig.11).

Poethke et al (122,125-127) have published a series of articles on the TLC of the opium alkaloids. The alkaloids were determined qualitatively and quantitatively, using two-dimensional TLC on alumina layers without binder (127). Vignoli et al (128) have used two-dimensional TLC on silica gel G, with the solvents methanol-chloroform-ammonia (85+15+0.7) and diethylether, saturated with water-acetone-diethylamine (85+8+7); they then identified the separate opium alkaloids by means of the different colours yielded with the iodoplatinate reagent.

Mary and Brochmann-Hanssen (129) quoted 11 solvents with which the most important opium alkaloids can be separated. Ethanol-benzene (20+80) good for papaverine and narcotine (cf. Fig.11).

The hR<sub>f</sub>. values of opium alkaloids on various layers, using solvents I-XVI are reproduced in Table 8 (124).

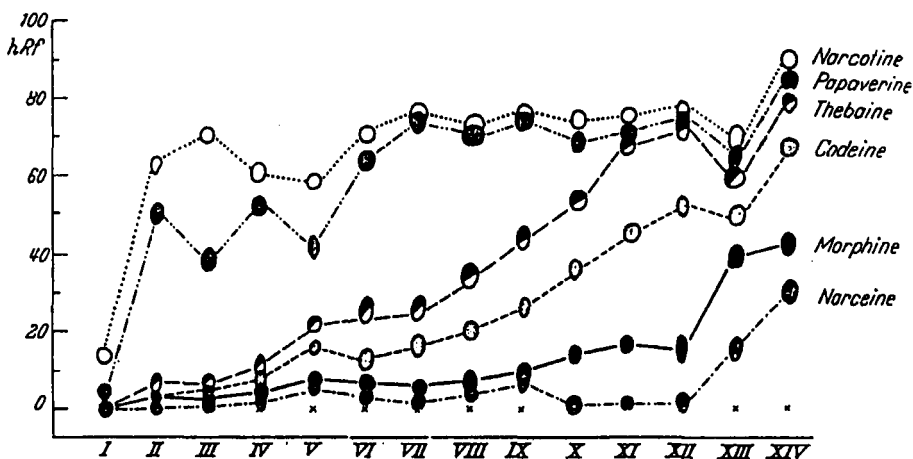


Fig. 11. Comparison of customary solvents for separating 6 important opium alkaloids. Layer: Silica gel GF<sub>254</sub>; chamber with CS; 20° C; 15 cm run (124)

No. Solvent, composition, time of run and references

- I. Benzene-tetrahydrofuran (95 + 5); 35 min
- II. Benzene-n-butanol (75 + 25); 30 min
- III. Benzene-tetrahydrofurane (80 + 20); 60 min
- IV. Benzene-n-propanol (80 + 20); 35 min
- V. Benzene-methanol (90 + 10); 30 min
- VI. Benzene-ethanol (80 + 20); 45 min
- VII. Chloroform-isopropanol (80 + 20); 45 min
- VIII. Chloroform-n-hexane-methanol (65 + 25 + 10); 30 min
- IX. Chloroform-methanol (90 + 10); 30 min analogous to
- X. Xylene-butanone-methanol-diethylamine (40 + 40 + 6 + 2); 35 min
- XI. Benzene-dioxan-ethanol-25% ammonium hydroxide (50 + 40 + 5 + 5); 45 min
- XII. Chloroform-acetone-diethylamine (50 + 40 + 10); 30 min
- XIII. Chloroform-acetone-methanol-triethylamine (30 + 40 + 10 + 20); 30 min
- XIV. Carbon tetrachloride-n-butanol-methanol-6N ammonium hydroxide (40 + 30 + 30 + 2); 90 min

Solvents I, IX, (149); II-VII (129);  
VIII (137); X (123);  
XI (132); XII (109);  
XIII (150); XIV (151)

Table 8. *hRf*-values of opium alkaloids on various layers, using solvents I—XVI (124)

Layer	S	S	S	A <sub>1</sub>	S 0.1 N NaOH	S	S	S 0.5 N KOH	C	C Form- amide	S	A <sub>1</sub>	S	A <sub>1</sub>	A <sub>1</sub>	A <sub>1</sub>
Solvent	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
Narceine	3	0	0	0	0	23	—	—	—	—	—	86	—	—	—	—
Dihydromorphinone (dilaudid)	24	23	8	8	16	28	5	13	27	6	14	—	—	—	—	—
Dihydrocodeinone (dicodid)	51	65	21	43	18	29	10	28	34	63	—	—	—	—	—	—
Morphine	10	8	0	0	34	40	2	2	27	0	24	14	12	3	3	6
Morphine ethyl ether (dionine)	—	—	—	—	—	37	14	37	44	57	—	72	—	—	—	—
Dihydrocodeine (paracodine)	38	54	18	30	25	—	6	22	34	—	—	—	—	—	—	—
Codeine	38	53	16	27	35	43	12	33	41	37	26	73	26	77	38	33
Acetyldihydrocodeinone (acedicone)	—	—	—	—	—	—	24	59	—	90	—	—	—	—	—	—
Dihydro-hydroxycodeinone (eucodal)	—	—	—	—	—	—	47	70	79	75	—	—	—	—	—	—
Thebaine	65	90	51	76	40	—	—	—	—	—	—	91	45	—	—	—
Papaverine	67	90	42	84	70	82	74	78	86	89	74	86	59	89	88	73
Cotarnine	60	90	43	25	0	—	—	—	—	—	—	—	—	—	—	—
Narcotine	72	90	51	79	72	82	78	81	92	94	69	90	74	92	77	77

*Layer:* S = silica gel G; A<sub>1</sub> = alumina; A<sub>2</sub> = alumina G; C = cellulose powder.

*Solvent:* I = Chloroform-acetone-diethylamine (50 + 40 + 10) ; II = Chloroform-diethylamine (90 + 10) ; III = Cyclohexane-chloroform-diethylamine (50 + 40 + 10) ; IV = Cyclohexane-chloroform (30 + 70) + 0.05% diethylamine ; V = Methanol ; VI = Methanol-acetone-triethanolamine (50 + 50 + 1.5) ; VII = Chloroform-ethanol (90 + 10) ; VIII = Chloroform-ethanol (80 + 20) ; IX = Dimethylformamide-diethylamine-ethanol-ethyl acetate (5 + 2 + 20 + 75) ; X = Benzene-heptane-chloroform-diethylamine (60 + 50 + 10 + 0.2) ; XI = Methanol ; XII = Benzene-ethanol (90 + 10) ; XIII = Xylene-butanone-methanol-diethylamine (20 + 20 + 3 + 1) ; XIV = Anhydrous acetone ; XV = Chloroform ; XVI = Benzene-chloroform-acetone (70 + 15 + 15)

Solvents: I, II, III, IV, V (109); VI (114); VII, VIII, IX, X, (110), XI (116); XII (124); XIII (123); XIV, XV, XVI (120).

In the quantitative determination, the spots were scrapped off the plate (silica gel G (Merck), eluted with methanol, the eluate made up to 5 ml and the concentration of alkaloid determined from the light absorption (w.l. used was 279 nm for papaverine) Steele (130) has recently achieved good separation of opiates in narcotic seizures, using silica gel G layers. The  $R_f$  values of 26 compounds were evaluated in 8 solvents, the best solvents were found to be ethylacetate-benzene-acetonitrile-ammonia (50+30+15+5) and acetonitrile-benzene-ethylacetate-ammonia (40+36+25+5).

Other articles in which the TLC of opium alkaloids or their pharmaceutically important derivatives described were reported (50,131-148). Visualization was achieved by inspection under UV at 254 nm, and by spraying with Dragendorff reagent or acidified iodoplatinate solution or dilute potassium iodobismuthate solution.

#### 8.8.3 Adsorption Chromatography

Mixtures of salts of organic bases, including papaverine, were separated by chromatography on silicic acid, with buffer solutions as eluents. A nomogram for selecting the correct pH of the eluent was constructed (152). Papaverine-HCl and quinine-HCl were separated at pH 4.6, papaverine-HCl and atropine-HCl at pH 7.2.

#### 8.8.4 Gas Liquid Chromatography (GLC)

Several GLC methods have been developed for papaverine among major opium alkaloids (153-158). Similar GLC procedures are in use for biological fluids, toxicology, and illicit drug screening (159-163). Most of the procedures use silylated or acetylated derivatives for the opium alkaloids.

Papaverine was separated by GLC by using 3 ft x 0.07 in. glass column packed with Gas Chrom P. The packing was washed with conc HCl, dried, and treated with HMDS, and coated with 1% of a cyanosilicone, a polyester methylsilicone copolymer, and cyclohexanedimethanol succinate polyester (154).

Using gas chromatography, papaverine contained in opium extract among other alkaloids, have been identified and quantified as a free base in one step without derivatization (164). An internal standard is used for the evaluation (165). For this purpose, accurately weighed amounts of opium extract and internal standard are prepared and an aliquot part of the resultant solution was subjected to GC:

Column : Support : Chromosorb G-HP AW-DMCS 80/100  
mesh : Stationary phase : 0.75% H1-EFF 8 BP,  
Dimensions : Length 1.5 m, i.d. approx. 2.3 mm  
Detector : FID Conditions of Separation :  
Temperature : injection block 270°C, Detector  
300°C. Oven 150 to 235°C, 1.25°C/min, Gases :  
carrier gas 40 ml/min., H<sub>2</sub> 50 ml/min., Air  
290 ml/min.  
papaverine retention time = 6.09 min.

A rapid gas chromatographic method for the simultaneous and quantitative determination of papaverine in gum opium has been developed (166). The opium alkaloids are completely separated (Fig. 12) on a column packed with a 50:50 of OV-17 and SE-30. The standard deviation of the method for the individual alkaloids is between 0.05 and 0.18%. The method was performed as follows:

The method depends on extraction of total opium alkaloids with water containing isoascorbic acid and sodium hydrosulfite. This solution was salted out with sodium chloride and the pH adjusted to 8.8 with ammonia and the liberated bases were extracted with 15% isopropanol in CHCl<sub>3</sub>. After removal of the solvent, the residue was dissolved in a certain volume of methanol-chloroform (25:75) containing the internal standards didecyl phthalate and resmethrin. The percentage of papaverine was calculated from a standard solution prepared and analyzed under the same conditions.

A glass spiral chromatographic column 6 ft. in length, 0.08 in i.d., packing material 50:50 mixture of 3% OV-17 on 80-100 mesh Varaport 30 and 5% SE-30 on 80-100 mesh chromosorb W., injector temperature : 310°C, oven temp. programmed, at 250° for 5 min. followed by increasing the temperature to 280° at a rate of 48°/min.,

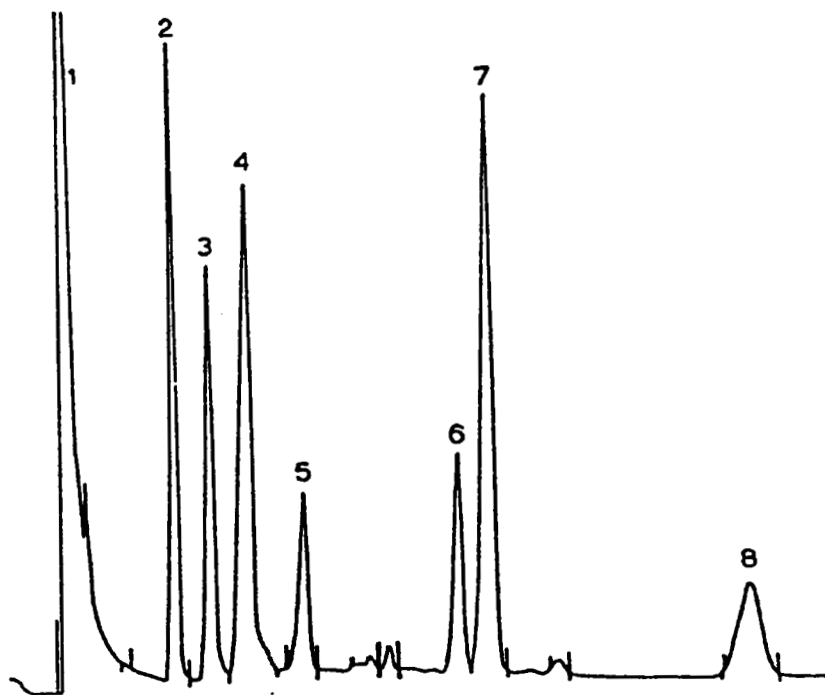


Fig. 12 GLC analysis of opium alkaloids. 1= Solvent; 2= resmethrin (internal standard) ( $t_R$ = 2.72 min); 3= codeine ( $t_R$ = 3.51 min); 4= morphine ( $t_R$ = 4.25 min); 5= thebaine ( $t_R$ = 7.63 min); 6= vapaverine ( $t_R$ = 9.15 min); 7= didecyl phthalate (internal standard) ( $t_R$ = 9.80 min); 8= narcotine ( $t_R$ = 15.65 min).

detector temp. 270°C flow rate of carrier gas (helium), 30 ml/min., H<sub>2</sub> pressure, 10 p.s. i.g. air pressure, 40 p.s.i.g.

A dimethyl sulfoxide solution of the opium sample was applied to a column of celite from which codeine, thebaine, papaverine and noscapine were eluted as one fraction, with a water-saturated mixture (7:3) of ethyl ether-light petroleum (b. range 30-60°C). These alkaloids were separated by GLC at 240°C on a column (6 ft x 4 mm) of 3% OV-1 on Gas-Chrom. Q (100-120 mesh) with N<sub>2</sub> as carrier gas (70 ml/min) and F.I.D. (167).

A recommended GLC method by the European pharmacopoeia Commission for determination of papaverine among opium alkaloids was reported (168). It involves extraction into a chloroform-propan-2-ol mixture from a dispersion of the sample in saturated aqueous sodium chloride solution at pH 10, filtration of the organic extract through anhydrous sodium sulfate, evaporation of the filtered extract to dryness and, after taking up the residue in ethanol and again evaporating to dryness, solution of the residue in the internal standard solution was used for the analysis: temp. programming 210-230°C on a column of 3.5% SE-30 on chromosorb W, with FID and histapyrrodine HCl or lynoestrenol as internal standard.

Two GC methods for the determination of papaverine in blood or plasma, which often is as low as 10 ng per ml, after injection of a therapeutic dose have been reported (169,170). In one method, a selective ion. pair extraction step to clean up the sample from interfering peaks was necessary (169) and the 2nd, an internal standard was added only after the extraction steps were completed (170). Also, the blood levels measured using an electron-capture detector were those achieved after administering twice the therapeutic dose level.

Another method for the determination of papaverine in blood samples using FID and NPD was described (171). The assay procedure was as follows: Blood or plasma (3.0 ml), internal standard (1.0 ml), water (1.0 ml) and 10.0 N KOH (1 ml) were pipetted into a 50-ml centrifuge tube equipped with a PTFE stopper and shaken for about 30

sec. on a vortex mixer. Toluene (10 ml) was added and the tube shaken vigorously for 10 min on a reciprocating shaker set at high speed. The tube was centrifuged and toluene extract transferred to a second 50-ml centrifuge tube. The extraction was repeated with a second 10-ml portion of toluene. The two toluene extracts were combined, 1.0 N hydrochloric acid (3 ml) was added and the toluene was extracted by shaking for 10 min. and then centrifuged. The organic phase was removed carefully, 1 ml of 10.0 N KOH was added and mixed. After cooling, 4 ml of ether was added and the tube was shaken for 2 min. on a vortex mixer, then centrifuged. The ether extract was transferred into a 15-ml centrifuge tube and treated with 0.5 g of anhydrous sodium sulfate. The dried extract was decanted into a 5-ml Reacti-Vial and the solvent was evaporated either by leaving the samples at room temperature overnight or using a water bath at 40°C and a stream of dry nitrogen. The residue was dissolved in 20 µl of isopropyl alcohol and 30 µl (for the FID) or 1.0 µl (for the NPD) were injected into the gas chromatograph. Standards were prepared by adding 1.0 ml of each working standard solution to 3.0 ml of blood or plasma. These standard were processed concurrently with the samples. The standards were prepared as follows:

- a) Using the FID : An amount of papaverine HCl equivalent to 50 mg of free base was dissolved into 50 ml water. This stock solution was then diluted with water to yield concentrations of 250,500 and 1000 ng/ml (working standard solutions). The internal standard solution was prepared by dissolving 40 mg of strychnine in 5-ml of ethanol and diluting to 50-ml with 0.1 N hydrochloric acid. This solution was further diluted with water to yield a concentration
- b) Using the NPD : Above stock solution of papaverine HCl was diluted with water to yield concentrations of 20,40,80 and 160 ng/ml of free base (working standard solutions). Similarly the internal standard solution was diluted to a concentration of 100 ng/ml.



Operating Conditions:For FID:

Column, glass, 1.2 m x 3 mm i.d. packed with 3% OV-17 on Gas-Chrom Q (80-100 mesh), injector and detector temperatures were 300 and 320 respectively. Flow rates of carrier gas (N<sub>2</sub>), H<sub>2</sub> and air were 80,40 and 300 ml/min respectively. The column was silylated in situ by injecting 15 l of Sylon-HTP and also treated with a concentrated alcoholic solution of papaverine and strychnine prior to any series of analysis.

For NPD, a glass column 1.2 m x 2 mm i.d. packed with 2% OV-101 on Chromosorb WHP (100-120 mesh) was used. The injector and detector temperatures were 300°C, the carrier gas (helium) flow-rate was 30 ml/min, plasma gases flow-rate (8% hydrogen in helium) was 30 ml/min., and the air flow-rate was 50 ml/min. The voltage for the alkali metal bead was set at 16. The column was treated with a solution of papaverine and strychnine as described above, but the use of silylating agent was avoided.

The measurement of papaverine in blood samples by using either a glass capillary column with an FID or a packed column with mass fragmentographic detection was described (172). A 3-ml volume of heparinized whole blood was treated with 5 ml of 0.12 N NaOH solution and the papaverine was extracted quantitatively with two 10-ml volumes of diethylether. The drugs were back-extracted in 5 ml of 0.1 N HCl and 4 ml of this phase made alkaline with 0.5 ml of 1 N NaOH solution was extracted with two 10-ml volumes of diethylether. Papaveraldine (400 ng), the working standard choosen for its structural similarity to papaverine, was then added to the organic phase. The solvent was slowly evaporated to dryness and the residue, re-dissolved in 3 ml of diethyl ether, was then transferred into a capillary tube sheltered from light. The evaporation was carried out carefully under a stream of nitrogen. The drug residue was finally re-dissolved in 50 l of chloroform and 1-5 l of the solution obtained was injected into the chromatographic column and the analysis was performed as follows:

- A) GLC on a packed column : The glass chromatographic column (2 m x 3 mm i.d.) was packed

with OV-17 (3%) on 100-120 mesh Gas-Chrom P(AW-DMCS). The gas vector flow was set at 40 ml/min - and the oven temp. at 272°. The temps. of the injector and the FID detector was 300°.

- B) GLC on a glass capillary column : The pyrex capillary column, (30 m x 1 mm o.d x 0.25 mm i.d.) was treated and deactivated according to the reported techniques (173,174). A static coating containing 0.8% SE-30 in n-hexane was applied (175). The introduction of the samples into the column was achieved by means of a solid injector device (176). The temperature of the chromatographic oven was 225° and that of the injector and FID detector cell was 280°C. N<sub>2</sub>, serving as the carrier gas, was regulated at Ca 1.5 ml/min for an inlet pressure of 1.3 bar. In order to use the F.I.D. under the optimal conditions, the authors claimed 30 ml/min of make-up nitrogen to be introduced at the base of the detector.
- C) Mass fragmentography : Specific ions of papaverine and papaveraldine were detected by a mass spectrometer equipped with a multiple ion detector. The glass chromatographic column was filled with 1% OV-1 on 100-120 mesh Gas-Chrom P and used under the same temperature conditions as those for the glass capillary column. The temp. of the separator was set at 290° and that of the source at 310°. The helium flow-rate was 32 ml/min, which corresponds to the minimum of the Van Deemter curve. The ionization current was 60  $\mu$ A and the electron energy 20 eV.

The authors came to a conclusion that measurements with a FID on a packed column were impossible because the papaverine peak was obscured by a large non-volatile impurity peak resulting from the extracting solvent. High-resolution Capillary column chromatography allowed the separation of the papaverine peak from those of the impurities. Nevertheless, the number and the importance of the background peaks and their qualitative and quantitative variations as a function of the solvent batch interfered with the measurements. Using the coupled GC/MS and a multiple ion detection device, the technical

problems were solved and a precision of about 2% was obtained. The limit of detection for papaverine in blood (2-500 ng/ml) was largely compatible with the therapeutic levels of this compound in blood.

On the other hand an article reported the identification of five papaverine metabolites in human urine (177) and another one recognized and gas chromatographically identified 50% of orally given papaverine in urine (178).

Procedures were described for the detection and measurements of 8 opium alkaloids including papaverine and metabolites in urine of opium eaters by methane chemical ionization mass fragmentography (179). The compounds were extracted from urine with  $\text{CH}_2\text{Cl}_2$  : isopropanol (7:3) and derivatized with Tri-sil X. The derivatives were subjected to GLC on glass column packed with 3% OV-210 on Gas Chrom. Q and temperature programmed from 170 to 260°C, with methane chemical ionization mass spectrometer, detection using selective ion monitoring.

The following analytical conditions were carried out in our laboratory for the GLC analysis of papaverine:

Column 3% OV1 on Chromosorb W., Temperatures : analysis from 250-300° by a rate of 10°/min; Detector, FID at 250°, Injector, 250°C, Carrier gas,  $\text{N}_2$  with 40 ml/min flow rate,  $\text{H}_2$  flow, 30 ml/min, air flow, 300 ml/min. Retention time for papaverine was 9.4 min. (Fig.13).

Other methods for measurement of papaverine in biological fluids were also discussed (180,181).

#### 8.8.5 High Performance Liquid Chromatography (HPLC)

The use of HPLC for quantitative analyses of pharmaceuticals has been increasing rapidly. In 1973 Wu et al (182) successfully demonstrated that HPLC technique is potentially useful for separation and analysis of opium alkaloids.

Gradient HPLC System was claimed (183) to be suitable for the determination of opium alkaloids.

A procedure for HPLC analysis using normal

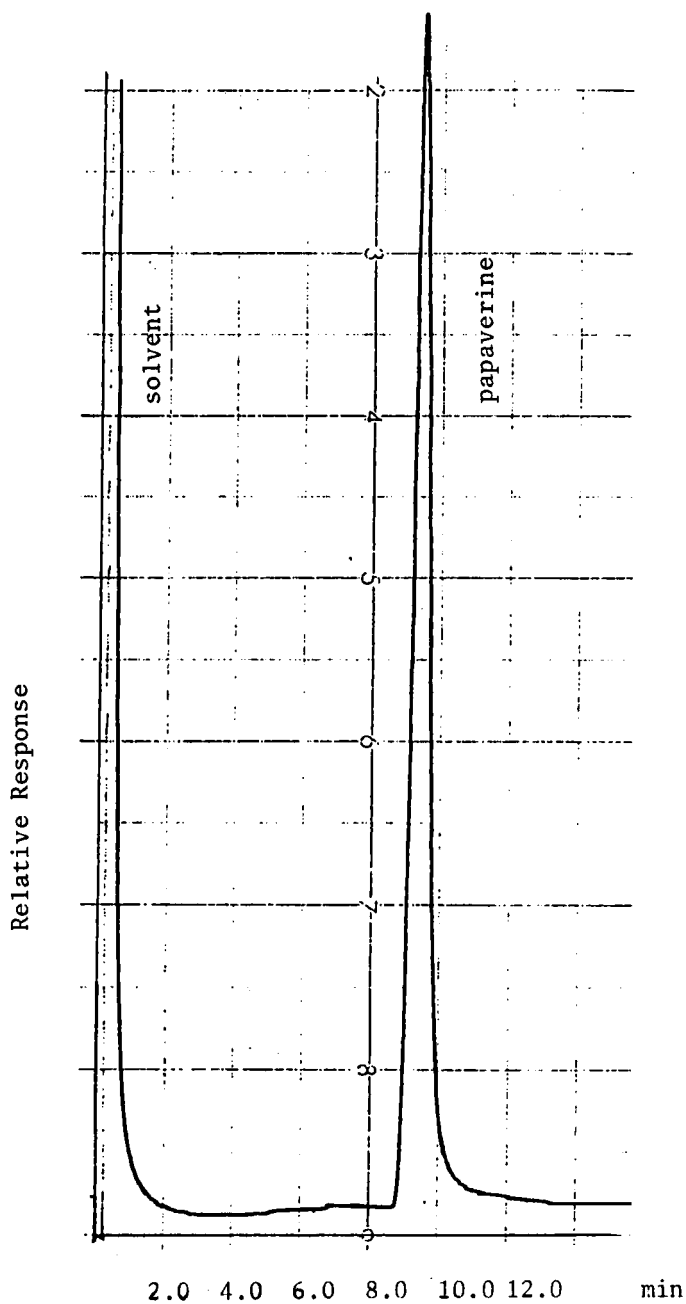


Fig. 13. GLC chromatogram of papaverine  
 $t_R=9.4$  min.

phase liquid-solid adsorption chromatography with gradient elution was described (184). This method, however, was reported to be lengthy and, unlike reverse phase chromatography, separation by normal phase liquid-solid adsorption chromatography is highly susceptible to the water content both of the adsorbent and of the mobile phase (185) making the technique difficult to use for routine analysis.

Performance and selected applications of a new range of chemically bonded packing materials in HPLC separation of different opium alkaloids including papaverine were reported (186). The following analytical conditions were followed: Column packing, SAS-silica (6  $\mu$ m); column dimensions 125 mm x 5 mm i.e.; eluent, 0.025 M  $\text{NH}_3$  in MeOH  $\text{H}_2\text{O}$  (1:1); pressure, 8000 p.s.i; temperature, ambient, detector, UV at 240 nm.

An HPLC procedure for separation of five major alkaloids including papaverine in gum opium was discussed (187). A microparticulate,  $\mu$ -Bondapak  $\text{C}_{18}$  column was selected and used because it provided the high column efficiency which is necessary for the analysis of a complex mixture such as gum opium. The authors stated that this column also provided excellent reproducibility over a long period of time, making it especially suitable for routine analysis. Chromatographic conditions for separation of the major opium alkaloids in a standard mixture (Fig.14) were as follows: Solution : A mixture of five standard alkaloids in 10 ml mobile phase (morphine sulfate 11.4 mg, codeine alkaloid 5.5 mg, thebaine alkaloid 7.2 mg, papaverine hydrochloride 5.0 mg, noscapine alkaloid 10.0 mg).

Mobile phase : 0.1 M  $\text{NaH}_2\text{PO}_4$  in 25%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  pH 4.8 Detector : 254 nm UV, Flow rate : 1.25 ml/min. Pressure : 2000 psig. In opium, the sample preparation was carried out by weighing two grams of the sample which was allowed to soak overnight in 20 ml of water. The resulting slurry was completely dispersed by stirring and by ultrasonic agitation. The supernatant was separated by centrifugation and transferred to a labeled 50-ml volumetric flask 1. The solids were thoroughly washed two more times using 15- and 10-ml portions of water and, after centrifugation, the supernatants were added to flask 1.

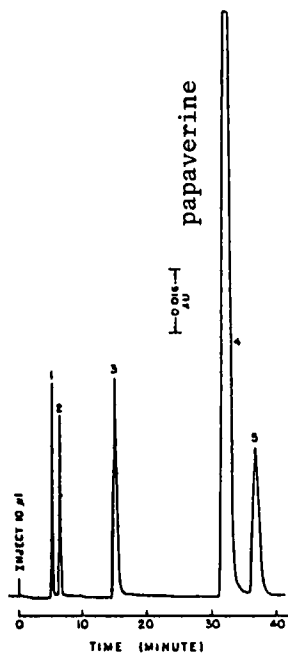


Figure 14. Separation of five of the major opium alkaloids with pH 4.8 mobile phase

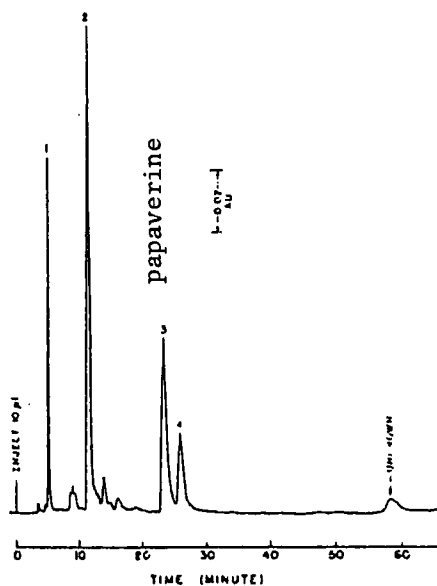


Fig. 15. Typical chromatogram of methylene chloride opium extract after being evaporated and redissolved in the mobile phase

The solution in flask 1 was diluted to volume with water before being used in subsequent analyses. A 25-ml aliquot of the aqueous opium extract from flask 1 was extracted in the presence of 0.2 g of calcium hydroxide powder and 5 g of sodium chloride with 10.0 ml of methylene chloride. After phase separation using high speed centrifugation, a 4.0-ml aliquot of the methylene chloride phase was diluted to 25-ml with presaturated methylene chloride and mixed. The entire methylene chloride solution was backwashed with 20 ml of presaturated  $\text{Ca}(\text{OH})_2$  solution and filtered through a Millipore Mitex filter using a syringe fitted with a Swinney adapter. Exactly 15.0 ml of the filtrate was evaporated to dryness and then redissolved in 10.0 ml of the mobile phase to be used with the aid of a few drops of 85% phosphoric acid to ensure complete solution. This solution was filtered through a Millipore Fluoropore filter and analyzed chromatographically. Fig.15 depicts a typical chromatogram of methylene chloride opium extract after being evaporated and redissolved in the mobile phase.

The effect of pH of mobile phase on retention of the five alkaloids was recorded. For papaverine, at pH 2.0 it was eluted at 18.5 min., at 4.8 at 33.0 min. and at 7.1 at 60 min. PH at 4.8 was found the most suitable and used throughout the proposed procedure (187).

Application of reverse phase ion-pair partition chromatography to drugs was described for determining papaverine (188). A single isocratic system and a fixed wavelength (254 nm) UV detector were used. Paired-ion chromatography was performed on a reverse phase  $\mu$  Bondapak C<sub>18</sub> column. A counter ion, 1-heptane sulfonate, was dissolved in the aqueous organic mobile phase to give a final pH of approximately 3.5.

An HPLC isocratic procedure was described for determining and quantitating the 5 major alkaloids, narcotine, papaverine, thebaine, codeine, and morphine (189) in papaver somniferum. The authors stated that the advantages of this method is that no precolumn or other purification other than solvent extraction of the capsular tissue is necessary. Isocratic chromatography alone on a single column resolved the

5 major alkaloids.

A rapid and sensitive HPLC assay was described for the quantification of papaverine in plasma (190). A microparticulate reversed-phase HPLC column, a fixed-wavelength UV detector and a potentiometric recorder was used for separation and quantification. The system was operated at ambient temperature with a flow rate of 2.0 ml/min. and the detector was set at 254 nm. The mobile phase consisted of 55% (U/v) methanol, 1% (U/V) acetic acid, and 0.005 M 1-heptanesulfonic acid sodium. Prior to use, the mobile phase was degassed by vacuum filtration through a 5-  $\mu$ m filter. The procedure was carried out as follows: An aliquot of plasma, 1.0 ml, in a 15-ml glass-stoppered centrifuge tube was spiked with 2  $\mu$ g of chlorpheniramine maleate as the internal standard (100  $\mu$ l of an aqueous solution) and vortexed. A 0.2-ml aliquot of 7 N NaOH and 10.0 ml of ether were added. The sample was shaken mechanically for 15 min. and centrifuged to separate the phases. The ether phase was transferred with a Pasteur pipet to another centrifuge tube and extracted with 0.2 ml of 0.3 N HCl by shaking and centrifuging as described previously. The ether phase was aspirated, and 100  $\mu$ l of the aqueous phase was injected. The ratio of the peak height of papaverine to that of the internal standard was used to calculate the papaverine concentration, based on a calibration curve prepared from spiked plasma samples.

A simple and rapid method for the routine quantitative analysis of papaverine among the major alkaloids of gum opium by direct isocratic HPLC on a reversed-phase partition mode column, without using ion-pair reagents was described (191). A 2-g amount of gum opium was mechanically shaken with 20 ml of 2.5% acetic acid for 20 min. Then the mixture was centrifuged, and the supernatant was separated and filtered. The extraction procedure was repeated three times. The extracts were combined and made up to 100 ml with 2.5% acetic acid. A 5-ml volume of the aqueous acetic acid solution was diluted to 20 ml with methanol, and 6  $\mu$ l of the solution was injected into the liquid chromatograph (Fig. 16,17).



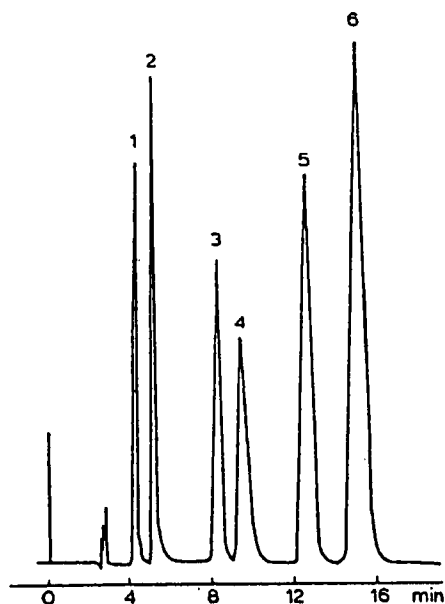


Fig.16 Chromatogram of six alkaloids on Nucleosil 10CN. Mobile phase, 1% ammonium acetate (pH 5.8)-acetonitrile-dioxane (80:10:10); flow-rate, 1.5 ml/min. 1 = Morphine; 2 = codeine; 3 = cryptopine; 4 = thebaine; 5 = narcotine; 6 = papaverine.

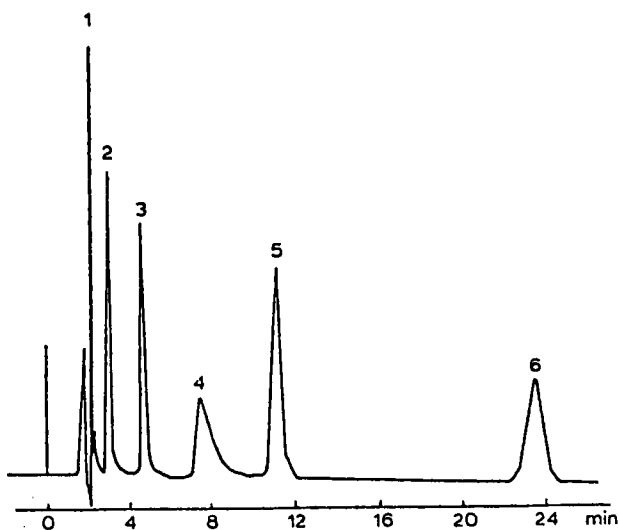


Fig.17 Chromatogram of six alkaloids on Nucleosil 10C<sub>14</sub>. Mobile phase, 1% ammonium acetate (pH 5.8)-acetonitrile (65:35); flow-rate, 1.5 ml/min. 1 = Morphine; 2 = codeine; 3 = cryptopine; 4 = thebaine; 5 = papaverine; 6 = narcotine.

**Chromatographic Conditions:**

Systems A-D : column, 300x4 mm I.D. Nucleosil 10 CN; Systems E-G : column, 300x4 mm I.D. Nucleosil 10 C<sub>18</sub>; Mobile phases : A, 1% ammonium acetate (pH 5.8)-acetonitrile-dioxane (80:10:10); B, 1% ammonium acetate (pH 5.8)-acetonitrile (80:20). C and E, 1% ammonium acetate (pH 5.8)-acetonitrile (70:30); D and G, 1% ammonium acetate-(pH 5.8)-acetonitrile (60:40); F, 1% ammonium acetate (pH 5.8)-acetonitrile (65:35). All systems : flow-rate, 1.5 ml/min.

Papaverine showed retention times (min.) 15.7, 18.2, 9.3, 5.6, 20.8, 11.3 and 8.0 in systems A to G respectively.

A C<sub>18</sub> (Li chrosorb RP8) stationary phase and 58 Methanol, 420.015 M sodium formate pH 8.5 as mobile phase were used for separation and quantification of papaverine (192).

The separation of morphine, codeine, noscapine and papaverine in reversed phase ion-pair chromatography has been investigated by means of statistical optimization methods (193). The value of the capacity factor, as a function of the methanol-water ratio. pH and the concentrations of buffer and ion-pair reagent, including their interactions, was studied for each compound. It was shown that, by using such methods, conditions could be chosen which essentially improved the separation and that a quantitative characterization of the optimum region was facilitated.

Papaverine concentrations in blood were measured by HPLC (52). To 1 ml of blood 1.5 ml of 1 M NaOH was added and the mixture was extracted twice with ether. Aliquots were evaporated by nitrogen and the residue was redissolved in 0.1 M HCl. Samples were injected in duplicate into a high pressure liquid chromatograph fitted with Mikro Bondapak C-18 column. The mobile phase was 30% acetonitrile in 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (pH = 5.0).

Another HPLC assay method for determination of papaverine in whole blood was described (194). Mepyramine was used as the internal standard with separation on a column of Micropak CN-10 eluted with a mixture of n.hexane, dichloromethane, acetonitrile and propylamine and U.V. at 254 nm for detection.

A reversed-phase HPLC method for the analysis of papaverine among the major opium alkaloids in poppy straw concentrate, using a phenyl type bonded phase was reported (195). Approximately 50 mg of sample were accurately weighed into a small erlenmeyer flask. To this were added 25 ml of solvent A containing 1 mg/ml of quinine sulfate as an internal standard. Solution was effected by sonication for 30 min. An aliquot of this solution was filtered through a Gelman Acrodisc-CR filter, pore size 0.45  $\mu$ m. Standards were dissolved in the same batch of solvent plus internal standard as the sample, and treated in the same manner. Injections were of 10  $\mu$ l. The column was a phenyl Bondapak, 25 cm x 5 mm I.D. and was used in conjunction with a 7 cm x 2 mm I.D. guard column packed with C<sub>18</sub>/Corasil, particle size 37-50  $\mu$ m. Solvent A was acetonitrile-water (5:95) and solvent B was acetonitrile-water (20:80). Both solvents contained 1 ml/l. of glacial acetic acid and 0.04 ml/l. of N,N-dimethyl-lactylamine. The pH of both was adjusted to 3.5 with sodium hydroxide. Degassing was accomplished by sonication under vacuum. The column was allowed to re-equilibrate for 10 min. between samples. The flow-rate was 1.0 ml/min. The column eluate was monitored at 275 nm. Standards were run at the start, in the middle and at the end of a sample series.

The linearity of the method for papaverine was 0.5-5.0% and the detection limit was 0.1%. The authors stated that their method is simple, reproducible and sensitive. Eleven runs (three standards, 8 samples) can be made in a normal day, and sample preparation was easily achieved during the 35 min. run time.

Good HPLC separation of papaverine when in a mixture with morphine, codeine and thebaine (196) was achieved using Nova-Pak C<sub>18</sub>, Radial-Pak cartridge, 8 mm x 10 cm. Mobile phase : A) 10 mM Potassium perchlorate, monobasic: 5 m M n.butylamine, pH 3.0 adjusted with perchloric acid B) Acetonitrile. Flow rate : 3 ml/min. Detector, UV at 280 nm.

Another method for HPLC analysis of papaverine using silica (spherisorb, 5  $\mu$ m) 25 cm x 5 mm i.d. and methanol: ammonium nitrate buffer solution (90:10) was reported (197). Other methods for HPLC investigation of papaverine in opium were also discussed (198,199).

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ANALYTICAL PROFILE OF PHYTONADIONE

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## PHYTONADIONE

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10. Toxicity

11. Dosage12. Pharmaceutical forms13. Methods of Analysis

## 13.1 Elemental Composition

## 13.2 Identification

## 13.3 Titrimetric Methods

## 13.4 Spectroscopic Methods

## 13.5 Chromatographic Methods

## 13.6 Bio-Assay Methods

AcknowledgementReferences

## PHYTONADIONE

### 1. History

The signs of vitamin k deficiency were first observed in connection with studies of cholesterol metabolism carried out in the Biochemical Institute of the University of Copenhagen during the years 1928-1930 (1-3). From 1929 onwards several investigators (4-7) had reported a bleeding tendency in chickens raised on artificial diets. The nutritional chick disease was characterized by sub-cutaneous, intra-muscular and abdominal hemorrhages, anemia, hemophilia, erosions of the gizzard and heavy mortality. In 1931 the hemorrhagic disease in chicks was described in details by McFarlane and his co-workers (8,9). In the meantime Holst and Halbrook (10) of California University had also observed the disease and found that it could be prevented by the fresh cabbage. In 1934 Dam and Schonheyder (11) produced hemorrhagic syndrome in chicks and showed that the disease was not caused by lack of any of the known vitamins. The same finding was also obtained by Halbrook (12) and he noted that the disease was prevented by 5% of dehydrated alfalfa or by an equivalent level of an ether extract of alfalfa leaf. In 1935 Dam (13) has reported that the disease resulted from a deficiency of a factor which was found in hog liver fat, hemp seed, tomatoes, kale and to a less degree in many cereals. This antihemorrhagic factor was localized in the fat-soluble, unsaponifiable, non-sterol fraction. Dam (14) regarded this factor as a new vitamin and proposed the name vitamin k (Koagulation Vitamin). The first communication about vitamin k was very soon confirmed by Almquist and Stokstad (15,16) who were the first to show that the vitamin can be formed by purification. In the following years vitamin k was studied intensively. The knowledge of its distribution in nature was extended (17,18). The concentrates of the vitamin from alfalfa leaf were prepared by column chromatography and molecular distillation (19). The first clinical trials of vitamin k, showing its ability to eliminate the coagulation defect associated with obstructive jaundice were carried out in 1937 and 1938 by oral ingestion of concentrates from alfalfa leaf (20). In 1939, Dam *et al* (21) and Doisy *et al* (22) isolated vitamin k from alfalfa leaf and called it vitamin  $k_1$ , to distinguish it from a substance called  $K_2$  which has been isolated from putrefied fish meal by Doisy *et al* (23). The pioneering and outstanding work of Dam and Doisy on vitamin k has been recognised by the joint award of the Nobel Prize.

## 2. Description

### 2.1 Nomenclature

#### 2.11 Chemical Names

- a) 2-Methyl-3-phytyl-1, 4-naphthoquinone (24)
- b) 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-1,4-naphthalenedione (24).
- c) 3-phytylmenadione (24).

#### 2.12 Generic Name

Vitamin K<sub>1</sub>

#### 2.13 Synonyms

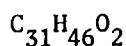
- a) Phylloquinone (25)
- b) Phytomenadione (25)
- c) Antihemorrhagic vitamin (24)
- d) Methylphytylnaphthochinonum (25)
- e) Phytomenad (25)
- f) Phytonadion (26)

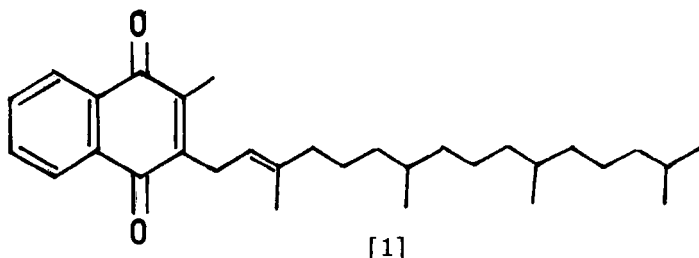
#### 2.14 Trade Names

- a) Aquamephyton (27).
- b) K-Ject (24)
- c) Konakion (27)
- d) Mono-kay (24)
- e) Mephyton (24)

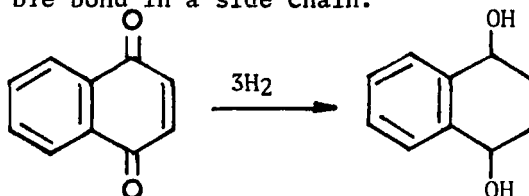
### 2.2 Formulae

#### 2.21 Empirical



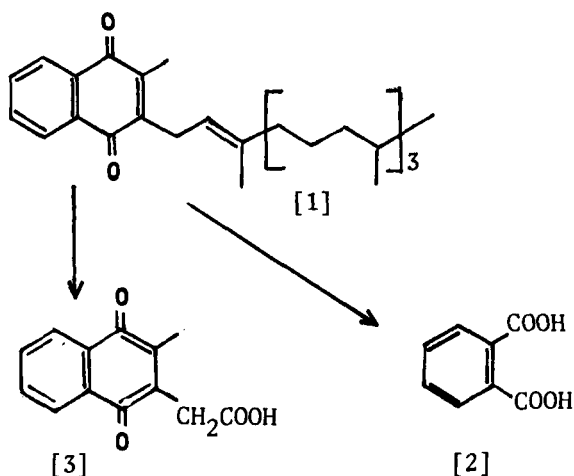
2.22 Structural

Vitamin K<sub>1</sub> is a light yellow oil, C<sub>31</sub>H<sub>46</sub>O<sub>2</sub>, which is a 1,4-naphthaquinone derivative.<sup>46</sup> The chemistry of vitamin K was established by the schools of Dam, Doisy and Karrer (28). The structure of vitamin K<sub>1</sub> has been proved by the study of its reaction and degradation products (29). The redox potential of vitamin K<sub>1</sub> is very similar to that of 1,4-naphthaquinones (21,30,31) and its ultraviolet spectrum is also similar to that of 2,3-disubstituted, 1,4-naphthaquinones (22). Thus vitamin K<sub>1</sub> appears to be 1,4-naphthaquinone derivative. The catalytic hydrogenation of vitamin K<sub>1</sub> causes the addition of four molecules of hydrogen (22), the product is a colorless compound. Since it is known that three molecules of hydrogen are added when 1,4-naphthaquinone is reduced under these conditions, the addition of a fourth molecule of hydrogen to the vitamin suggests the presence of an ethylenic double bond in a side chain.



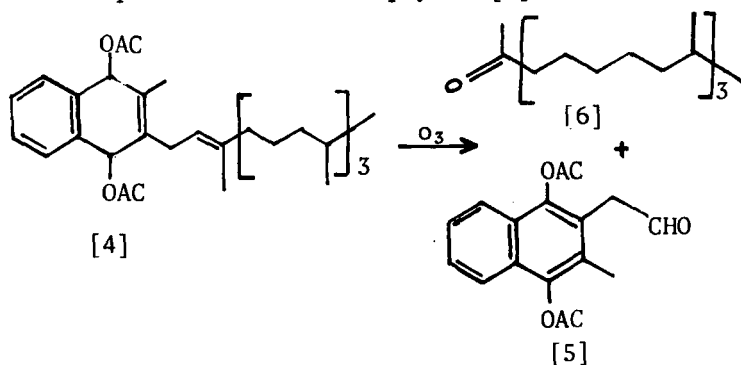
When subjected to reductive acetylation, vitamin K<sub>1</sub> [1] is converted into the diacetate of dihydro vitamin K<sub>1</sub> (22). This diacetate is difficult to hydrolyse. This is a property characteristic of 2,3-disubstituted 1,4-naphthaquinones. Chromic acid oxidation of vitamin K<sub>1</sub> [1] resulted in the formation of phthalic

acid [2] and 2-methyl-1,4-naphthaquinone-3-acetic acid [3] (32-35). A comparable acid was also obtained from the oxidation of diacetyl dihydro vitamin K<sub>1</sub> [4].



Thus the presence of 1,4-naphthaquinone is confirmed and at the same time these products show that one ring is unsubstituted and the other (the quinonoid ring) has substituents in 2- and 3- positions. This was also supported by the fact that the ultraviolet spectrum of vitamin K<sub>1</sub> showed very close similarity with 2,3-dialkyl derivatives of 1,4 naphthaquinone (36).

On ozonolysis the diacetate of dihydro vitamin K<sub>1</sub> [4] yielded the corresponding diacetoxy-acetaldehyde [5] and a C<sub>18</sub>-ketone [6] which was found to be identical with 6,10,14-trimethyl-2-pentadecanone (33,35) which is oxidation product of natural phytol [7].



Hence on the evidences obtained above vitamin K<sub>1</sub> is 2-methyl-3-phytyl-1,4-naphithaquinone [1]. The structure has been confirmed by total synthesis which was carried out independently by Doisy etal (34), by Almquist and Klose (37) and by Fieser (38).

2.23 CAS Registry Number

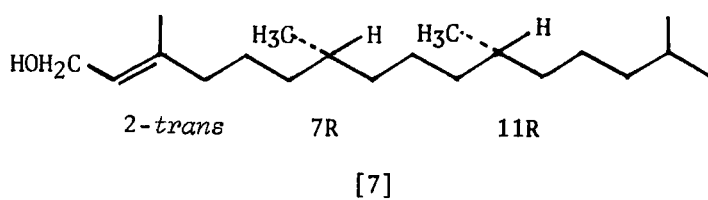
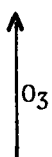
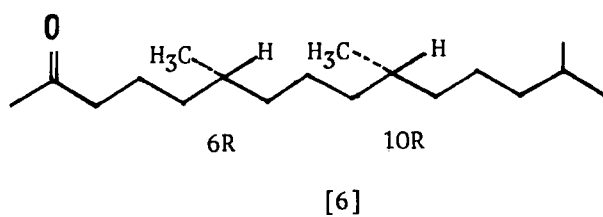
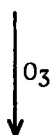
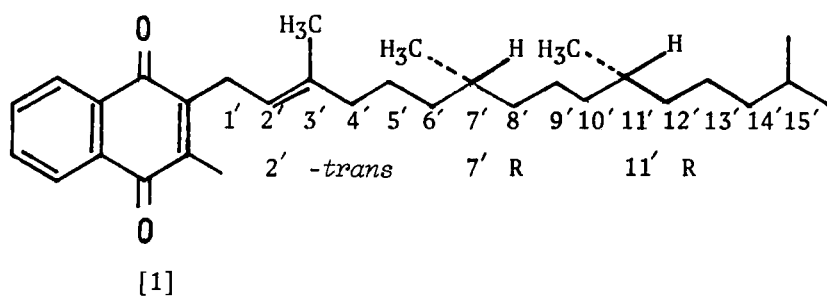
[84-80-0]

2.24 Wiswesser Line Notation

L66 BV EVJ C2U  
Y&3Y&3Y&3Y D (39)

2.25 Stereochemistry and Absolute Configuration

The stereochemistry and the absolute configuration of vitamin K<sub>1</sub> was well summarized by Mayer and Isler (28) and Finar (40). Inspection of the structural formula of vitamin K<sub>1</sub> [1] shows that two chiral centres are present (carbon atoms 7' and 11'), and that geometrical isomerism is possible about the 2',3'-double bond. In view of the fact that the vitamin has been synthesised from natural phytol, it can be expected that the two chiral centres in the vitamin would have the same configurations as those in natural phytol (7 and 11), i.e., 7'R and 11'R. This was confirmed by comparing the optical rotation and the ORD curve of the C<sub>18</sub>-ketone obtained by ozonolysis of vitamin K<sub>1</sub> with those of the C<sub>18</sub>-ketone from natural phytol [7] (41-43). The trans-configuration at the 2',3'-double bond corresponds to that in natural phytol, and this was confirmed by means of NMR spectroscopy (28,44). The absolute configuration of the natural vitamin K<sub>1</sub> is given below.





### 2.3 Molecular Weight

[450.70] (45)

### 2.4 Appearance, Color, Odor and taste

clear, yellow, very viscous liquid (oil); odorless or nearly odorless, stable in air, but decomposes, on exposure to sunlight. (24,26)

### 2.5 Acidity or Alkalinity

A 5% solution in dehydrated alcohol is neutral to litmus (25).

## 3. Physical Properties

### 3.1 Solubility

Insoluble in water. Soluble I in 70% alcohol; more soluble in dehydrated alcohol; freely soluble in chloroform, petroleum ether, benzene, acetone, hexane, dioxane and fixed oils (24,25).

### 3.2 Refractive Index

$n_D^{20}$  : 1.525 to 1.529 (26)

### 3.3 Specific gravity

The specific gravity is about 0.967 (24,46).

### 3.4 Optical Rotation

Measurement of optical rotations for vitamins K are difficult as the rotation are shown to be small. The following optical rotations were reported for vitamin K<sub>1</sub>:

$[\alpha]_D^\circ$	solvent	Ref.
$[\alpha]_D^{25} = -0.28^\circ$	Dioxane	(24)
$[\alpha]_D^{20} = -0.4^\circ$	57.5% Benzene	(39)

The optical rotation for phytonadione was determined in our laboratory on a Perkin Elmer polarimeter, Model 241 MC and found to be  $[\alpha]_D^{25} = -0.58$  in benzene ( $e = 1.0$ ).

### 3.5 Spectral properties

#### 3.5.1 Ultraviolet spectrum

The UV spectrum of phytonadione in cyclohexane was recorded from 200 to 400 nm on DMS 90 Varian AG spectrophotometer (Fig. 1). Five maxima were observed at 330, 270, 262, 249 and 247 nm.

Other UV data was also reported as follows (27, 36, 39, 47).

<u>Solvent</u>	<u><math>\lambda_{\max}</math> m<math>\mu</math></u>	<u><math>\epsilon</math> 1% 1 cm</u>
Iso-octane	327	69
	271	390
	263	385
	249	420
	245	398

#### 3.5.2 Infrared spectrum

The infrared spectrum of phytonadione as a film recorded on a Pye-Unicom 1025 infrared spectrophotometer is shown in Fig. 2. The functional group assignments have been correlated with the following bond frequencies (Table 1).

Table 1 : IR Characteristics of Phytonadione

<u>Band Frequency</u>	<u>Functional group</u>
cm <sup>-1</sup>	
1675	C=O stretching vibrations of quinone.
1632	C=C - stretching in quinone ring and in the phytyl side chain.

460

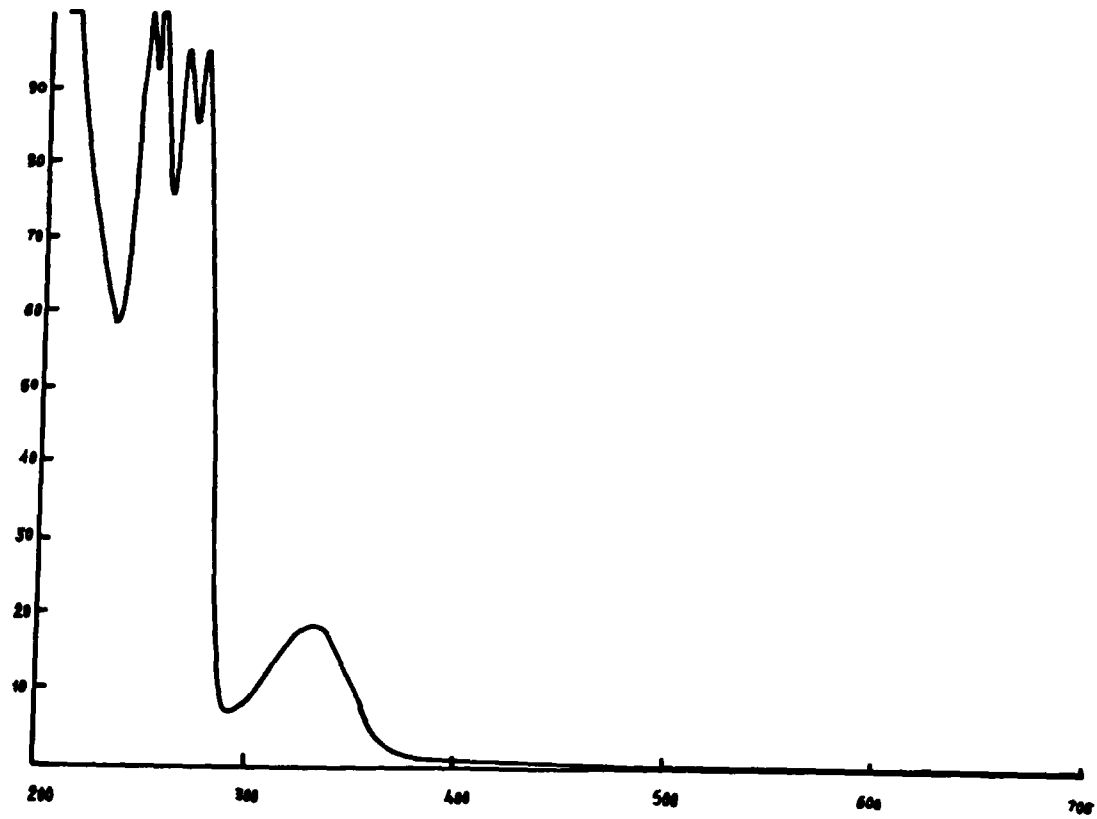


FIG.1 : UV SPECTRUM OF PHYTONADIONE IN CYCLOHEXANE.

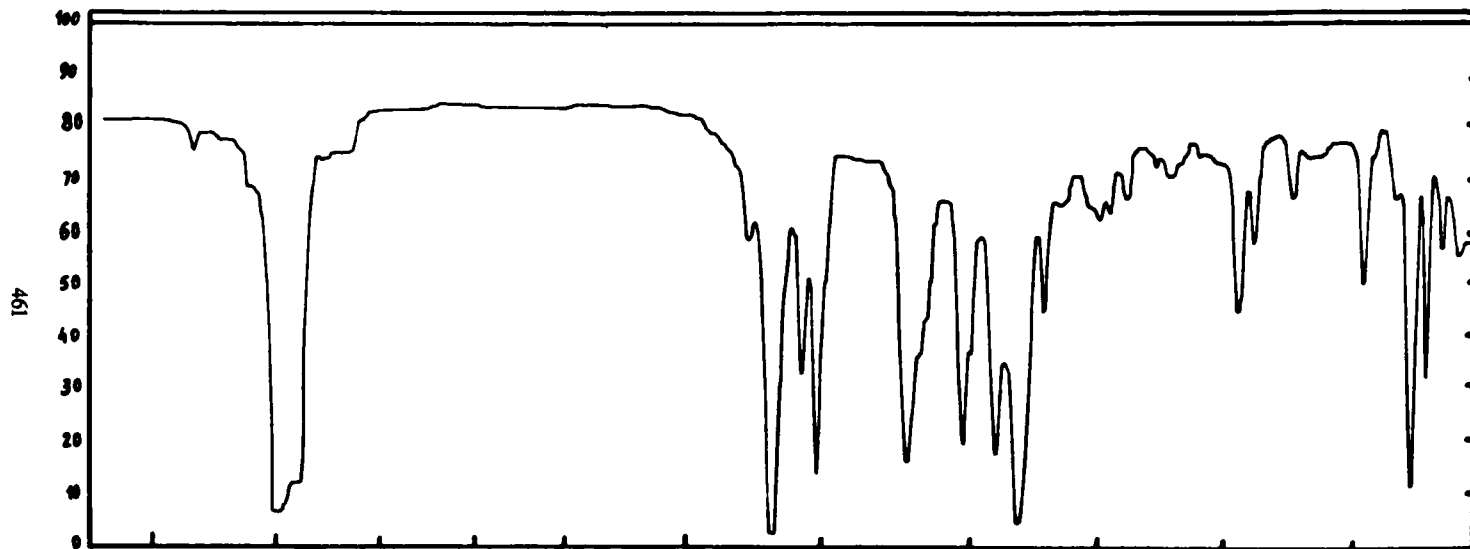


Fig.2: IR Spectrum of phytonadione as a flim.

<u>Band Frequency</u>	<u>Functional group</u>
1610	C=C stretching for $\Delta^{21}$ phytyl side chain and aromatic C=C.
700	C-H out-of-plane deformation
710	for the four adjacent ring
795	hydrogens.

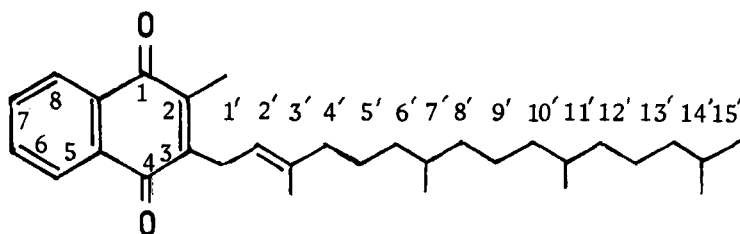
Other infrared spectral data were also reported (39,48).

### 3.5.3 Nuclear Magnetic Resonance Spectra

#### 3.5.3.1 $^1\text{H}$ -NMR spectra

The  $^1\text{H}$ -NMR spectrum of phytonadione in deuterated chloroform (Fig. 3) was recorded on FX-100-100 MHz FT-NMR (Jeol) spectrometer, with tetramethylsilane as an internal reference.

The proton chemical shifts are shown in table 2.



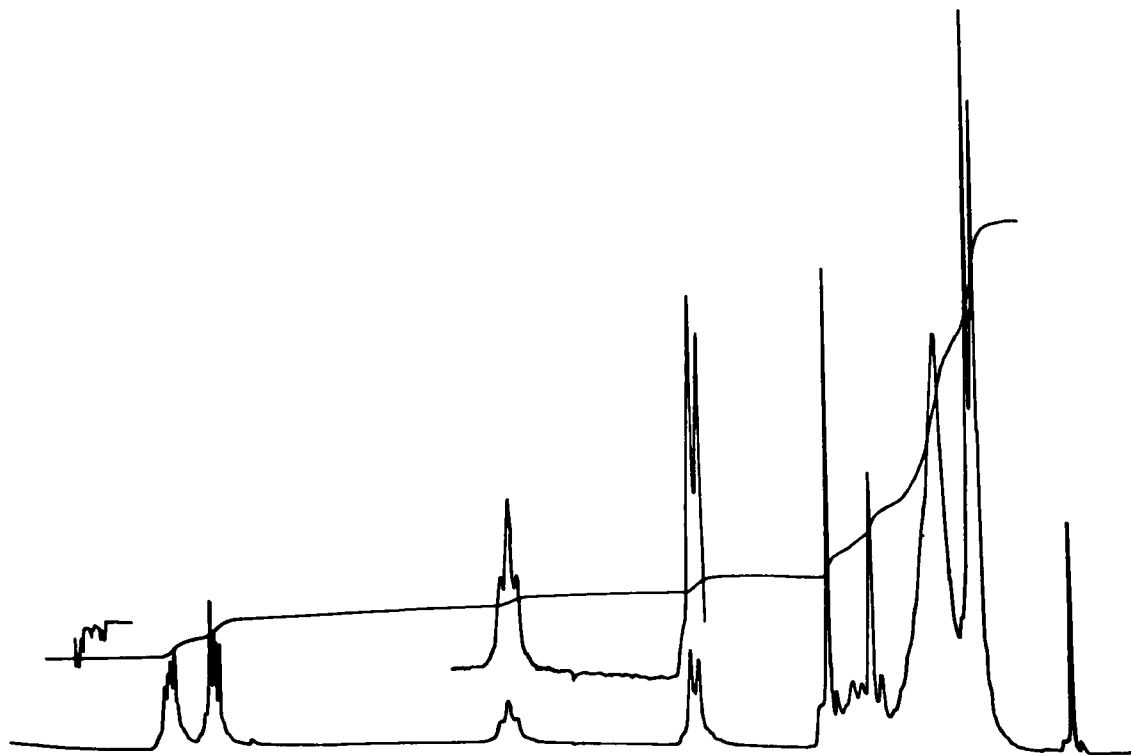


FIG. 3:  $^1\text{H}$ -NMR SPECTRUM OF PHYTONADIONE IN  $\text{D}_2\text{O}$  AND TMS

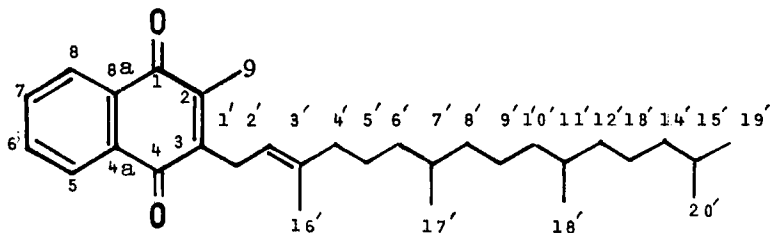
Table 2 :  $^1\text{H}$ -NMR Characteristics of Phytonadione

<u>Proton group</u>	<u>Chemical Shift <math>\delta</math> ppm</u>
5,8-H	8.40 (m)
6,7-H	7.98 (2d)
2'-H	5.28 (t)
1'-H	3.54 (d)
2-Me	2.31 (s)
3'-Me	1.86 (s)
Other protons of the phytyl side chain	1.26 (bs)
15-Me <sub>2</sub>	0.93 (d)

Other NMR data were also reported (28,48).

### 3.5.3.2 Carbon-13 NMR spectra

The natural abundance C-13 NMR noise-decoupled and the single frequency off-resonance decoupled (SFORD) spectra (Fig. 4 and Fig. 5) in deuterated chloroform were recorded on FX-100-25MHz FT-NMR (Jeol) Spectrometer using tetramethylsilane as reference standard. The carbon chemical shifts were assigned on the basis of the theory of chemical shift and SFORD splitting pattern (Table 3).



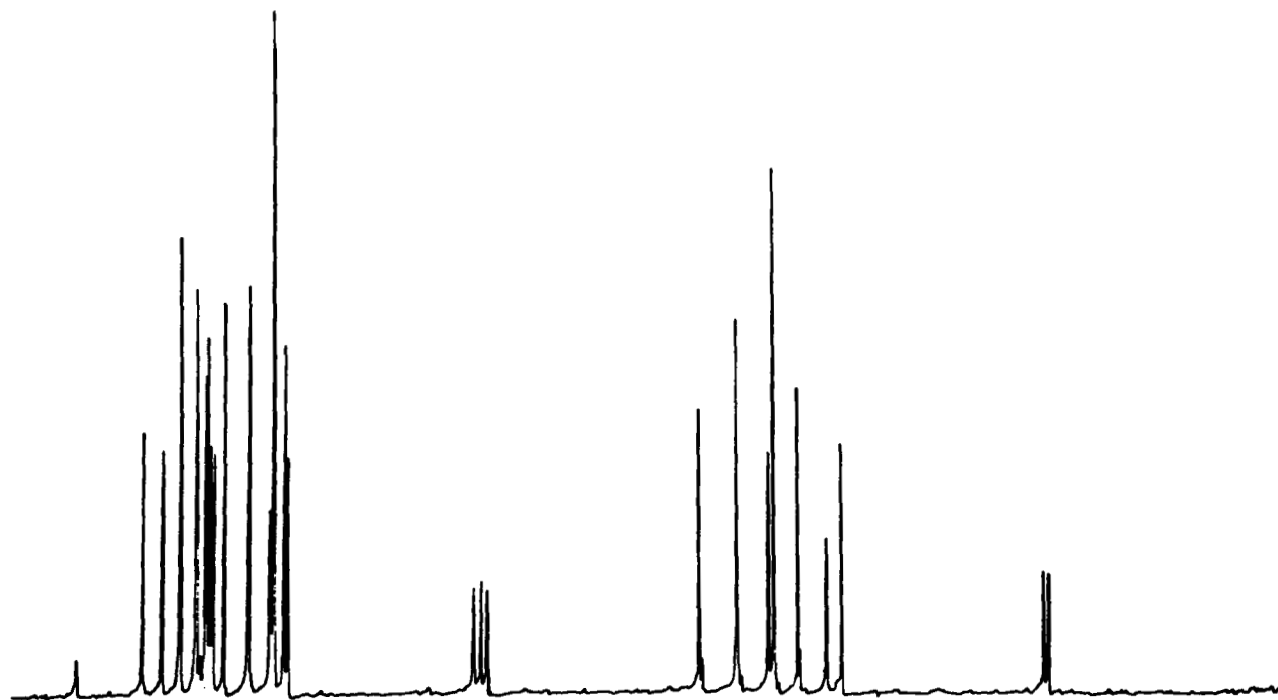


FIG.4 :  $^{13}\text{C}$ -NMR NOISE-DECOUPLED SPECTRUM OF PHYTONADIONE IN  $\text{D}_2\text{O}$  AND THS.



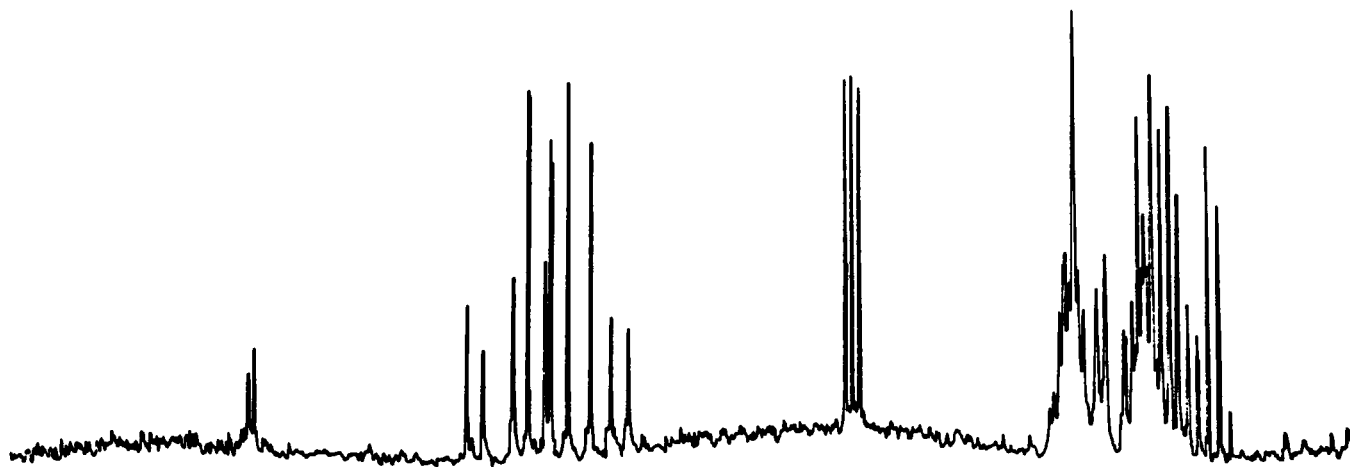


FIG. 5.  $^{13}\text{C}$ -NMR OFF-RESONANCE SPECTRUM OF PHYTONADIONE IN  $\text{D}_2\text{O}$  AND TMS.

Table 3 : Carbon-13 NMR Chemical Shifts of Phytonadione

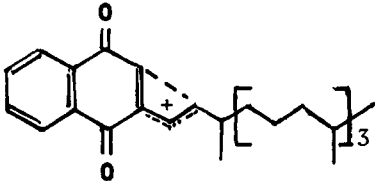
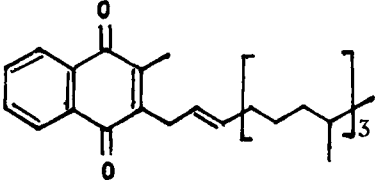
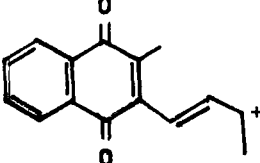
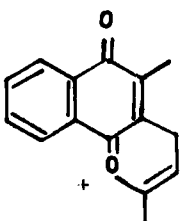
<u>Carbon No.</u>	<u>Chemical <math>\delta</math> ppm</u>	<u>Multiplicity</u>
1	185.06	s
2	146.01	s
3	143.12	s
4	184.14	s
4a	137.71	s
5	126.14	d
6	133.06	d
7	133.06	d
8	126.14	d
8a	137.71	s
9	22.60	q
1'	39.34	t
2'	118.89	d
3'	133.04	s
4'	27.94	t
5'	39.98	t
6'	37.34	t
7'	36.69	t
8'	37.34	t
9'	32.58	t
10'	37.34	t
11'	32.73	d
12'	37.34	t
13'	24.77	t
14'	37.34	t
15'	16.26	d
16'	12.59	q
17'	19.69	q
18'	19.69	q
19'	25.95	q
20'	25.24	q

### 3.5.4 Mass Spectrum

The mass spectrum of phytonadione obtained by electron impact ionization (Fig.6) which was recorded on a Finigam-Mat S100 mass spectrometer with an ionizing energy of 70 eV.

The most prominent fragments, their relative intensities and possible structures are listed in Table 4.

Table 4 : Mass Fragments of Phytonadione

<u>m/e</u>	<u>Relative intensity</u> %	<u>Fragment</u>
450	100	$M^+$ (base peak)
435	4.44	
		
225	65.49	
		

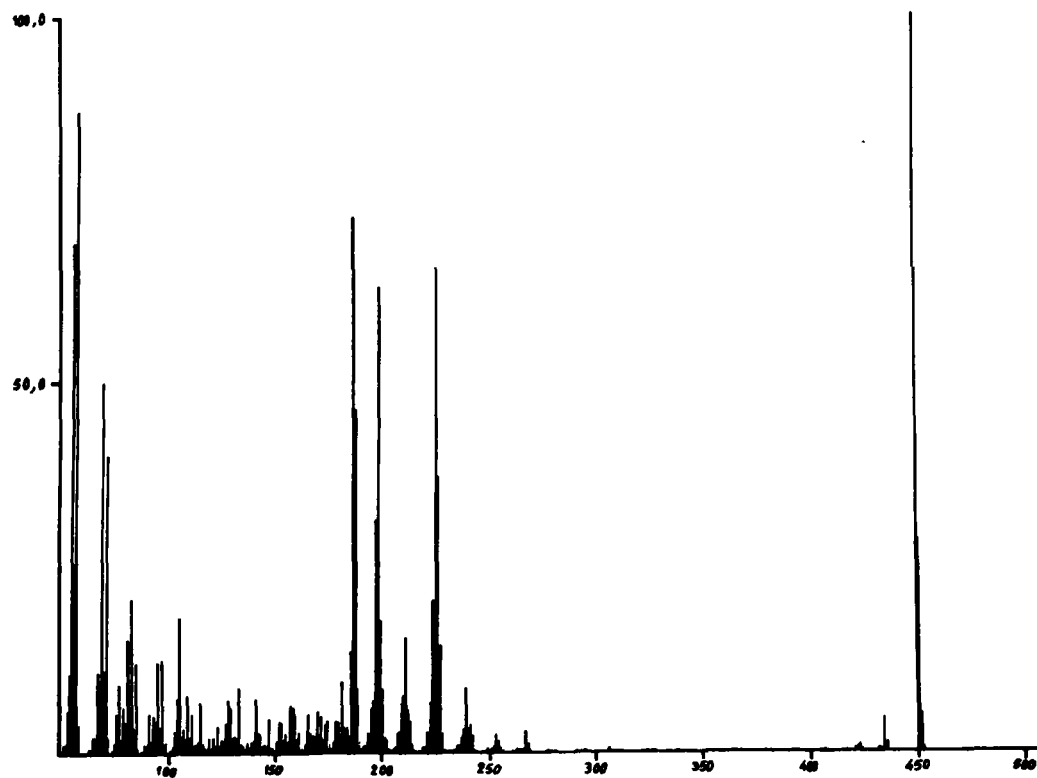
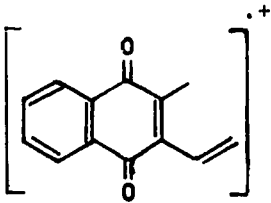
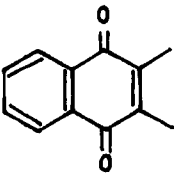
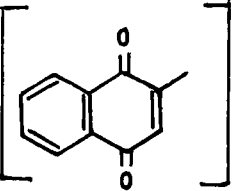
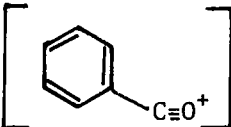
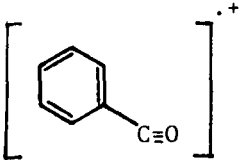
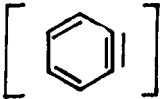


FIG. 6 : (E1) MASS SPECTRUM OF PHYTONADIONE.

<u>m/e</u>	<u>Relative intensity</u> %	<u>Fragment</u>
211	15.12	
198	62.88	
186	72.43	
171	4.67	
105	18.11	

<u>m/e</u>	<u>Relative intensity</u> %	<u>Fragment</u>
104	7.18	
97	12.50	$[\text{CH}_3(\text{CH}_2)_5\text{C}]^{\cdot+}$
83	20.60	$[\text{CH}_3(\text{CH}_2)_4\text{C}]^{\cdot+}$
76	5.44	
71	39.99	$[\text{CH}_3(\text{CH}_2)_4]^+$
69	50.00	$[\text{CH}_3(\text{CH}_2)_3\text{C}]^+$
57	87.02	$[\text{CH}_3(\text{CH}_2)_3]^{\cdot+}$
56	25.33	$[\text{CH}_3\text{CH}_2\text{CH}_1\text{CH}]^+$
55	68.91	$[\text{CH}_3(\text{CH}_2)_2\text{C}]^+$

Other mass spectral data of naphthaquinones and phytonadione were also reported (49).

#### 4. Preparation of Phytonadione

The vitamin K<sub>1</sub>, is widely distributed in greenish leafy materials, particularly in higher plants and in some blue-green algae (2,50-52). Alfalfa, Cabbage, hemp seed, tomatoe and kale are found to be relatively rich sources of this vitamin (10,12,13,30,53,54). The good heat stability of the vitamin (15) made possible the use of commercially dehydrated alfalfa meal as a starting source for the isolation of vitamin K<sub>1</sub>. The isolation of the vitamin K<sub>1</sub> from alfalfa was reported by several authors (22,30,31,55-57). A brief outline of the preparation of vitamin K<sub>1</sub> from alfalfa is well summarised below.

About 30 kg of artificially dried alfalfa powder is extracted with 40 L. of petroleum ether (40-60°) by hot percolation. The extract is then concentrated to about 20 L. and diluted with equal volume of petroleum ether. Finely powdered zinc carbonate (equal to 20 times the weight of dry matters in the extract) is added in small amounts with stirring. The mixture is agitated for 2 hours. If the supernatant liquid is still green, more zinc carbonate is added until the solution becomes reddish brown. An excess of zinc carbonate results in a loss of vitamin K<sub>1</sub>. After the zinc carbonate has settled down, the clear supernatant liquid is poured off, 5L petroleum ether is added to the zinc carbonate residue, the mixture is stirred and the supernatant liquid is again poured off. The residue is filtered and washed with petroleum ether until the washings are colorless. All the petroleum ether extracts are combined together, evaporated to about 500 ml and allowed to stand for 2 days at - 10°. The precipitated impurities is filtered off and washed with petroleum ether. The filtrate is then evaporated in vacuo on a water bath. A dark brownish red waxy residue containing 100,000-150,000 vitamin K units per gram is obtained. This residue is then subjected to molecular distillation. The portion coming off up to 150° containing practically all vitamin K, is collected. It is then liquified by warming slightly and then stirred with 3-4 times the amount of acetone. The undissolved solid portion is filtered off. The filtrate is kept in an ice box and then filtered again after 12 hours. The filtrate is evaporated to  $\frac{1}{2}$  of its volume and the resulting small crystals are removed. After evaporating the acetone completely in vacuo, a reddish brown oil containing the vitamin K<sub>1</sub> is left. The oily residue is dissolved in petroleum ether and passed through a column packed with magnesium sulphate. The solvent is evaporated and the residue is again subjected to a chromatographic separation. Different adsorbents have been used for

the final purification of vitamin K<sub>1</sub> (17,21,31,56).

The final purification of vitamin K<sub>1</sub> from the crude materials is achieved by the chromatography of the petroleum ether extract on silica gel and polyethylene followed by conversion of the concentrated material into its quinol diacetate, and further purification by chromatography and recrystallisation. The pure phyloquinone diacetate, upon reductive saponification and oxidation yield pure vitamin K<sub>1</sub> which is a yellowish viscous liquid.

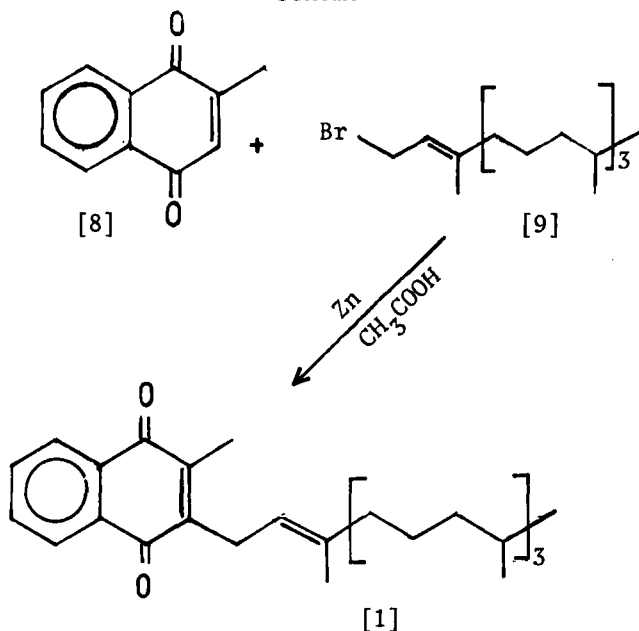
## 5. Synthesis of Phytonadione

### 5.1 Partial Synthesis

#### 5.1.1 From Menadione or Menadiol and Natural Phytol

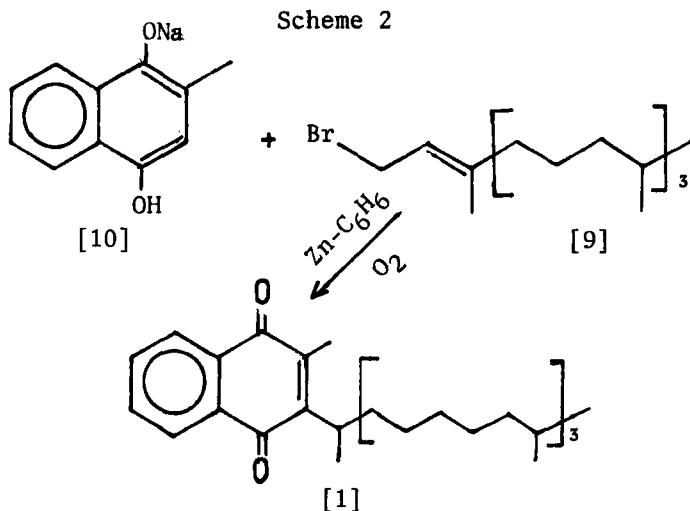
Synthesis of vitamin K<sub>1</sub> was accomplished by simple procedures. Three independent synthesis of vitamin K<sub>1</sub> were reported simultaneously in 1939, using menadione or menadiol and natural phytol or its derivatives. Almquist and Klose (37) synthesised vitamin K<sub>1</sub> [1] by condensing 2 methyl-1,4-napthaquinone (menadione) [8] with phytyl bromide [9] in the presence of zinc dust in acetic acid (scheme 1).

Scheme 1





Doisy et al (34,58) obtained vitamin K<sub>1</sub> [1] by condensing mono sodium salt of menadiol with phytol bromide [9] (scheme 2).



Fieser (38) obtained a better yield of vitamin K<sub>1</sub> [1] by condensing 2-methyl-1,4-naphtha quinol [1] with natural phytol [7] by oxalic acid in dioxane. The reaction product, dihydro vitamin K<sub>1</sub> [12] is oxidised to phytonadione [1] (scheme 3). A similar method of synthesis was also reported by Karrer et al (59).

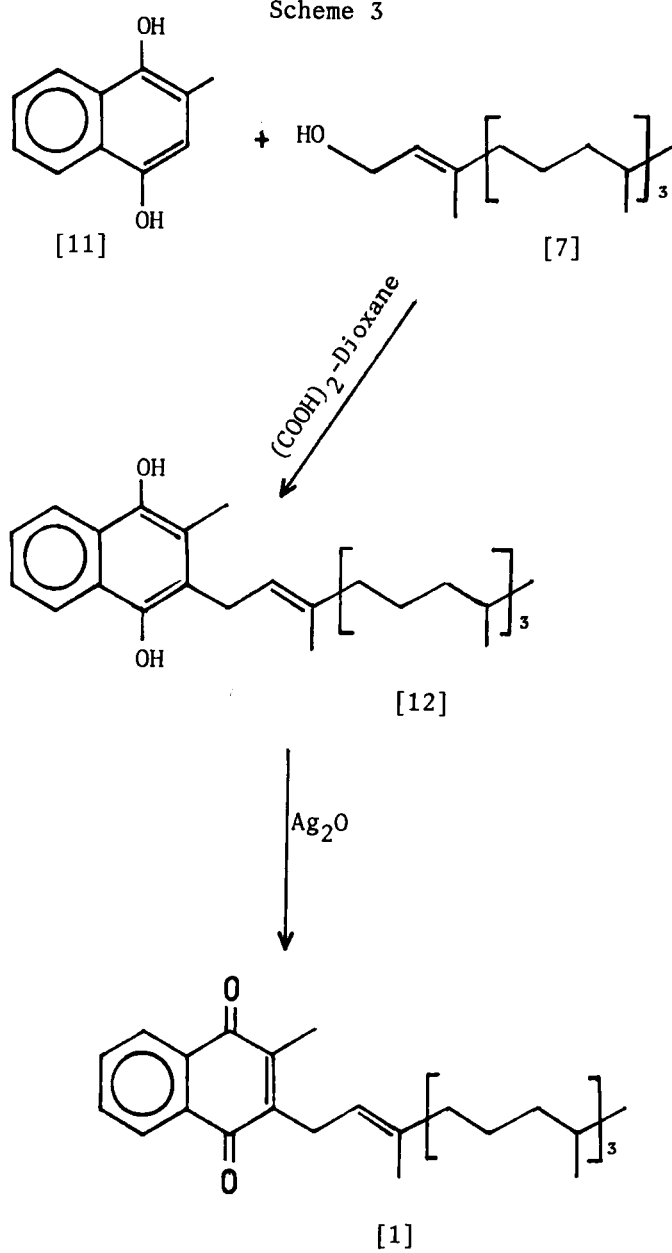
Synthesis of vitamin K<sub>1</sub> by condensing menadiol or menadione with phytol and its derivatives using zinc chloride, an acidic phosphate compound, sodium borohydride and silver oxide were also reported (60-62).

Jackman et al (44) synthesised vitamin K<sub>1</sub> [1], by Friedel-Crafts type condensation of menadiol [11] with *trans*-phytol [7] or isophytol [13]. The resulting intermediate hydroquinone [12] has been oxidised with silver oxide to yield *trans* phytonadione [1] (scheme 4).

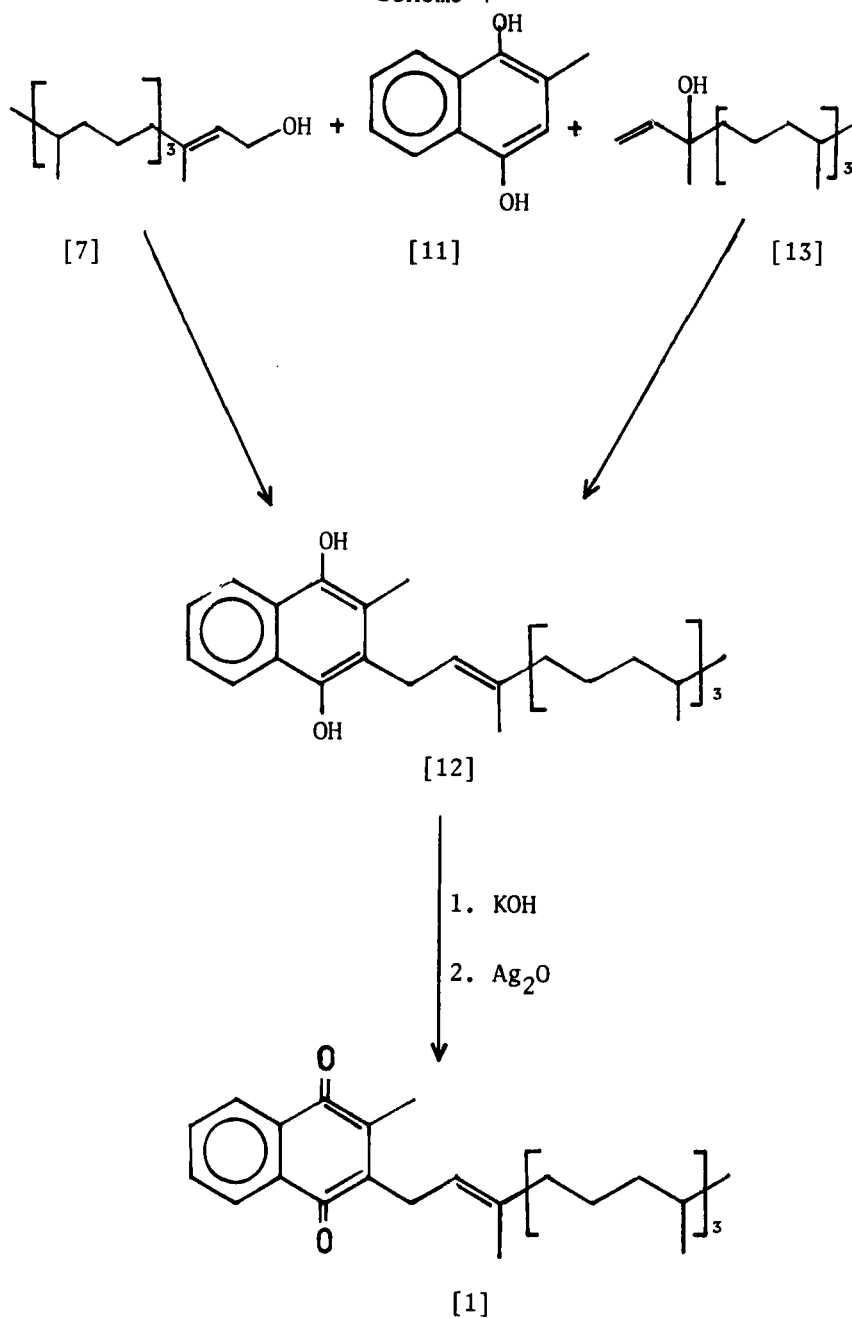
#### 5.1.2 Using $\pi$ -allylic nickel complex

Sato et al (63) have synthesised vitamin K<sub>1</sub> [1] using a  $\pi$ -allylic Complex [14]. Reaction of phytol bromide [9] obtained from natural phytol with excess of tetracarbonyl nickel in benzene under nitrogen at 52° for 4 hours

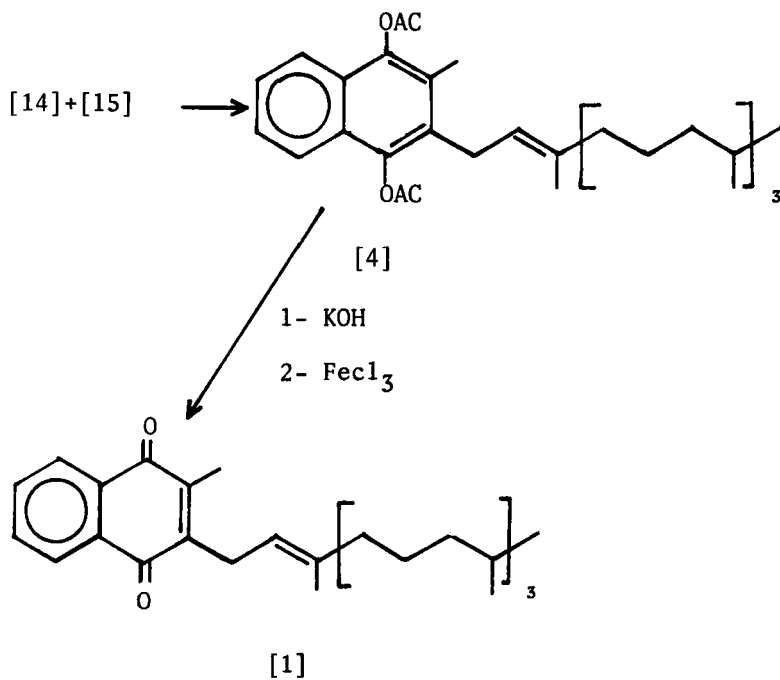
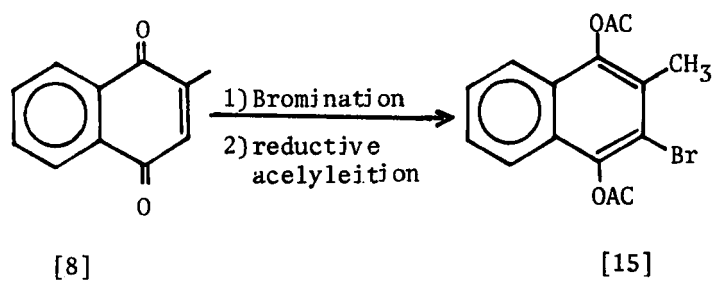
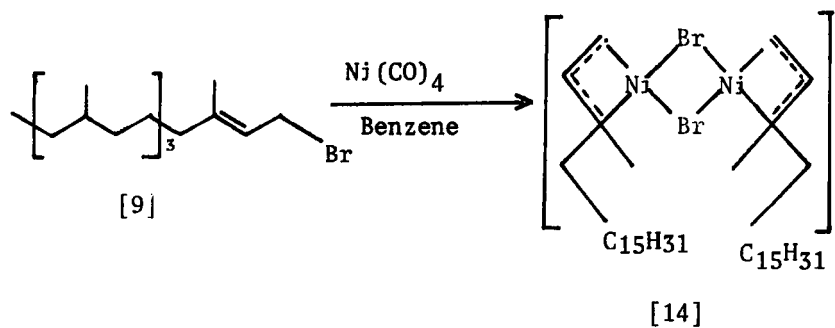
Scheme 3



Scheme 4



Scheme 5



afforded the phytyl nickel complex [14] which was treated with the diacetate of 2-bromo-3-methyl-1,4-naphthaquinone [15] which was obtained from menadione [8] in hexamethylphosphonamide and the resultant product was chromatographed on silica gel to give dihydro vitamin K<sub>1</sub> diacetate [4] which was hydrolysed with alkali and finally oxidised with ferric chloride to give pure vitamin K<sub>1</sub> [1] (scheme 5).

## 5.2 Total Synthesis

Sarycheva et al (64) synthesised phytonadione by using 2,6-dimethyl-2,6,8-undecatrien-10-one [16] as a starting material. The procedure is as follows:

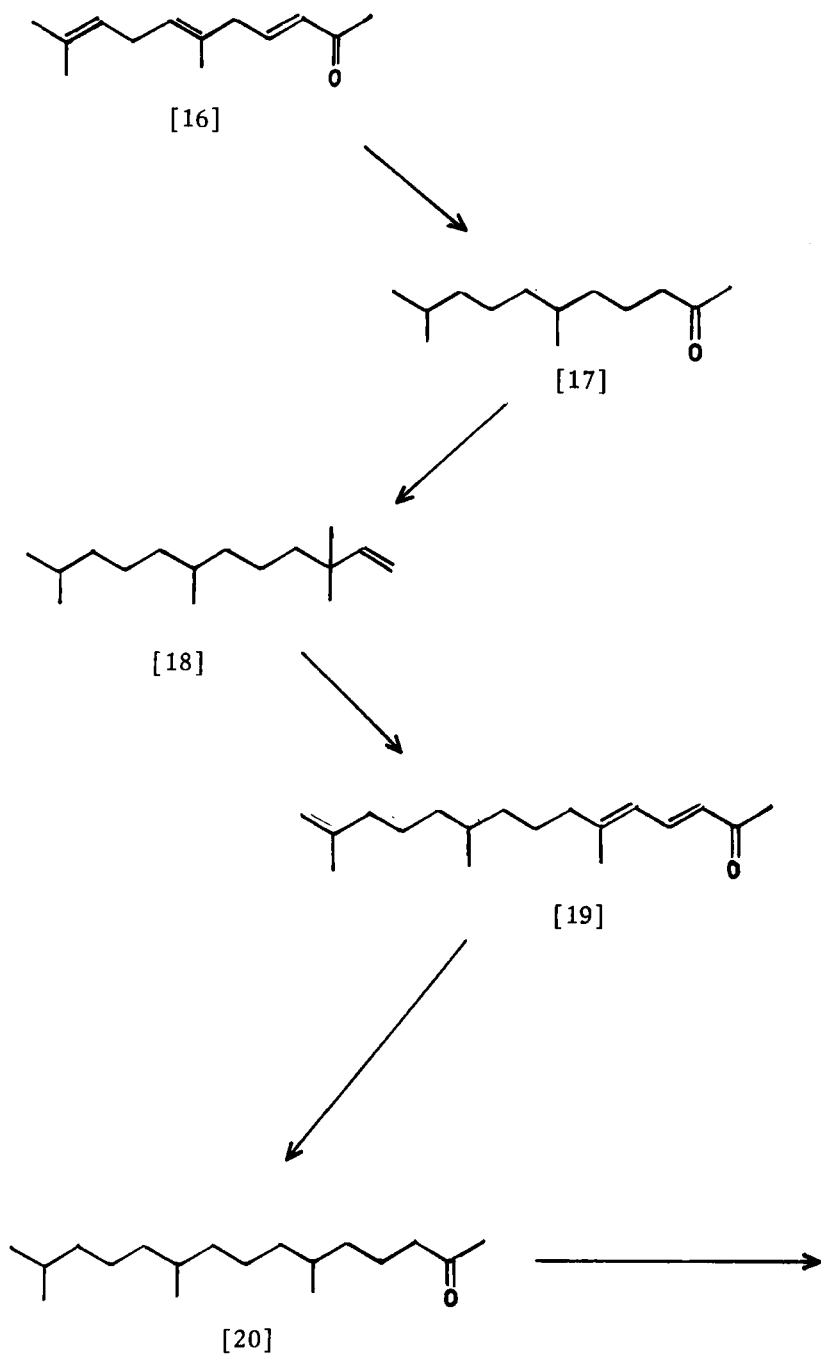
Hydrogenation of [16] in ethanol over Raney nickel, followed by oxidation with N potassium dichromate in acetic acid - sulphuric acid mixture and extraction with ether gave 2,6-dimethyl-10-undecanone [17]. This is added over to a solution of sodium in liquid ammonia. After treatment of [17] with a stream of acetylene, stirring for 12 hours and treatment with ice, gave 2,6,10-trimethyl-11-dodecyn-10-ol [18]. [18] is then heated with aceto ethyl acetate to give 2,6,10-trimethyl-10,12-pentadecadien-14-one [19]. [19] is then reduced and oxidised to 2,6,10,14-tetramethyl-15-pentadecanone [20] which with acetylene gave 2,6,10,14-tetramethyl-15-hexadecyn-14-ol [21]. Hydrogenation of [21] over palladium in petroleum ether gave 2,6,10,14-tetramethyl-15-hexadecen-14-ol [13] which is condensed with 2-methyl-1,4-naphthohydroquinone [8] in dioxane in the presence of boron trifluoride to give phytonadione [1] (scheme 6).

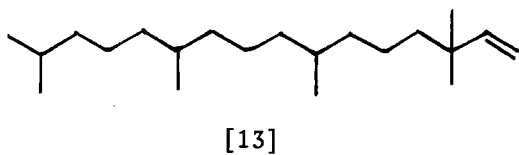
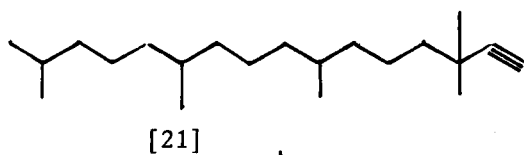
## 5.3 Commercial Synthesis

The yields in the early synthesis of vitamin K<sub>1</sub> [1] were very low and the purification of the products was rather difficult. Therefore, renewed synthetic methods have been employed in the industrial preparation of vitamin K<sub>1</sub> [1].

Hirschmann et al (65) has obtained vitamin K<sub>1</sub> [1] in good yield by condensing 1 acetyl derivative of 2-methyl-1,4-naphthaquinol [22] with phytol [7] in the presence of borontrifluoride. The condensation product, phylloquinone 1-monoacetate [23] is then saponified and the free quinol [12] is oxidised to vitamin K<sub>1</sub> [1] (scheme 7).

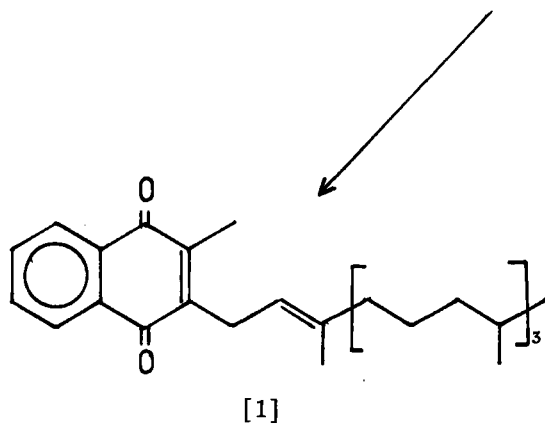
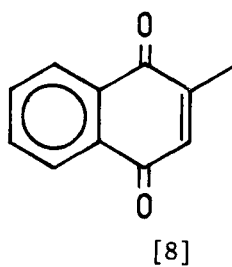
Scheme 6



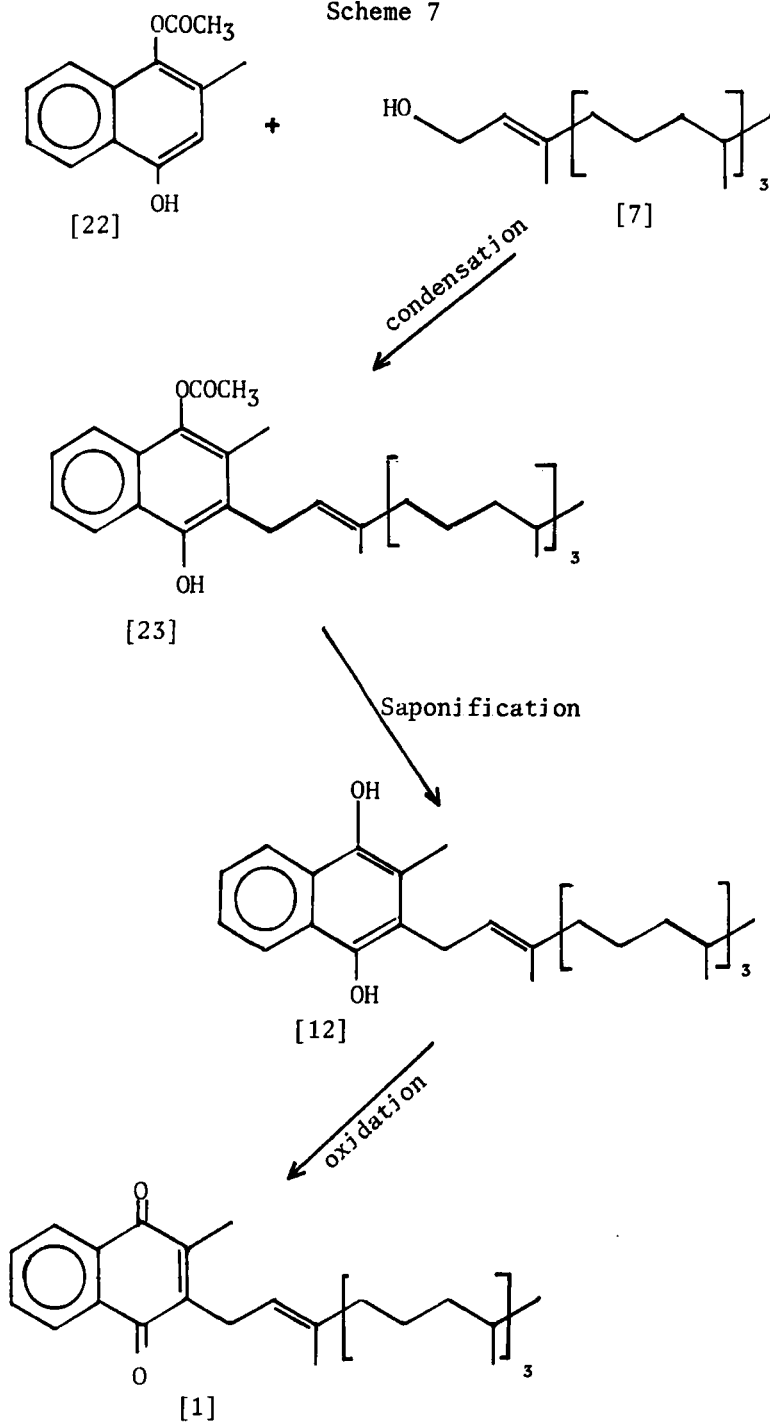


[13]

+

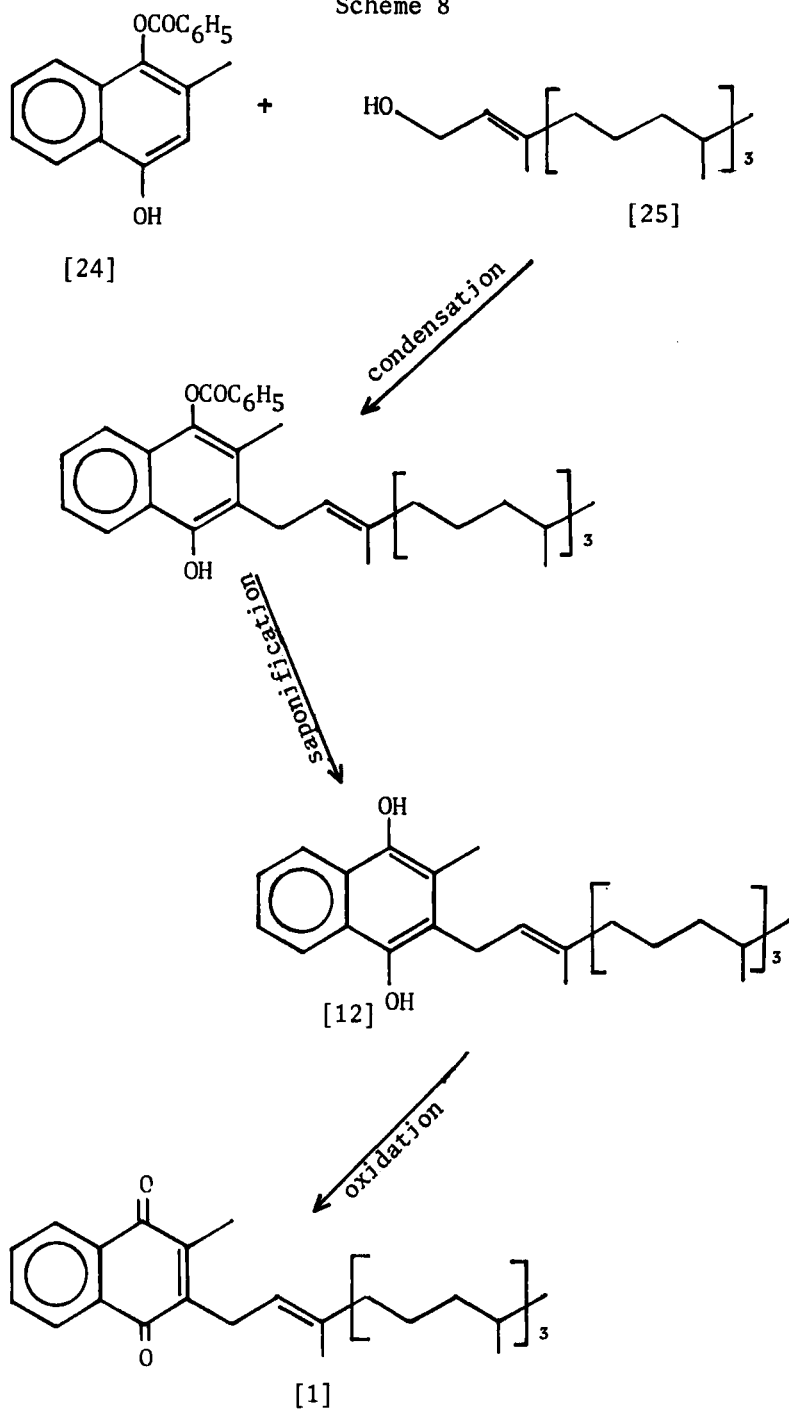


Scheme 7





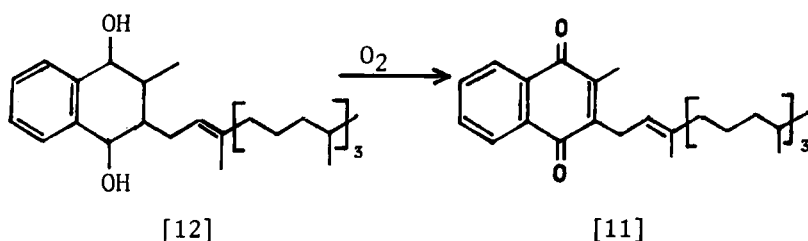
Scheme 8



Lindlar (66) utilized menadiol-1-benzoate [24] as a starting material for the production of vitamin K<sub>1</sub> [1]. Menadiol-1-benzoate [24] is condensed with phytol [7] using boron trifluoride as a catalyst to give phylloquinol-1-benzoate [25] which is saponified to quinol [12] and then oxidised to vitamin K<sub>1</sub> [1] (scheme 8).

Seiichi et al (67) have synthesised vitamin K<sub>1</sub> [1] from dihydro vitamin K<sub>1</sub> [12]. Dihydro vitamin K<sub>1</sub> [12] is oxidised with dimethylsulfoxide to give vitamin K<sub>1</sub> [1] (scheme 9).

Scheme 9

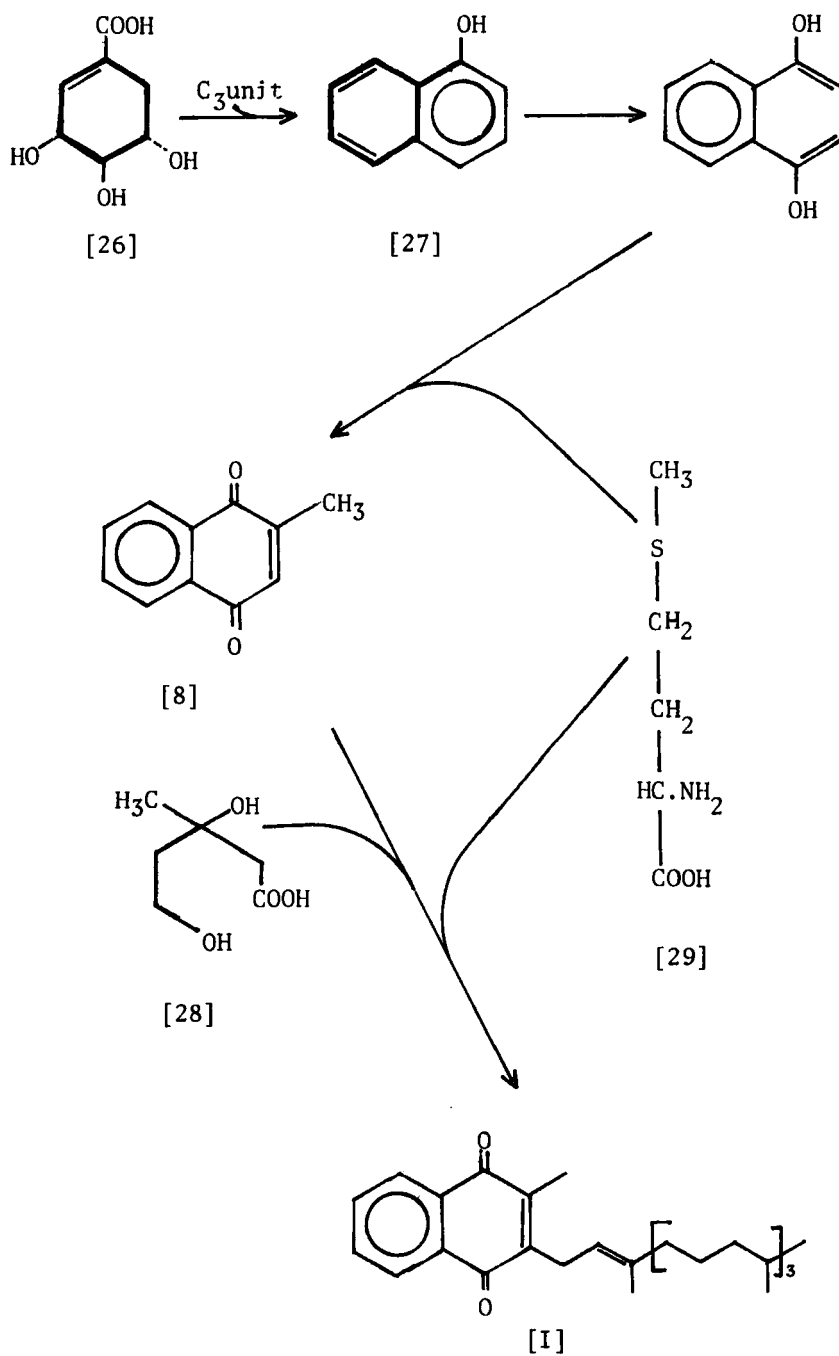


## 6. Biosynthesis of Phytonadione

The elucidation of biosynthesis of vitamin K<sub>1</sub> is not as far advanced as that of the plastoquinones, ubiquinone and to copherols, probably for the following reasons. The amount of vitamin K<sub>1</sub> present in plants is much smaller than that of the other isoprenoid quinones, thus rendering investigations more difficult. Moreover the knowledge of the biological role and biochemical function of vitamin K is still rather limited. Threlfall and Whistance (68) have described the biosynthetic study of vitamin K<sub>1</sub> in maize shoot, bean shoots and Ivy leaves. Seedlings of *picea canadensis*, eteolated maize and barley shoots show increase in the content of vitamin K<sub>1</sub> after illumination (18,69-71). These facts suggest the chloroplasts to be the cell organelles in which the vitamin K<sub>1</sub> is biosynthesised.

Whistance et al (72) and Whistance and Threlfall (73) found that on administration of shikimic acid-<sup>14</sup>C or shikimic acid-1,2-<sup>14</sup>C to green - excised- eteolated maize shoots, radioactivity was incorporated into naphthaquinone nucleus of phytonadione. Therefore shikemic acid [26] is assumed to be a precursor for the

Scheme 10



vitamin K<sub>1</sub>. Sasarman et al (74) suggested that menadione [8] also serves as a precursor for vitamin K<sub>1</sub>. Recently  $\alpha$ -naphthol [27] was claimed to be a precursor for naphthoquinone nucleus of menadione from *Bacillus megaterium* based upon incorporation of radioactivity from <sup>14</sup>C- $\alpha$ -naphthol by a whole-cell preparation (75).

Threlfall et al (76,77) and Datta et al (78) have reported that the polyprenyl side chain of vitamin K<sub>1</sub> is formed from mevalonic acid [28]. However the mechanism of interaction of mevalonic acid [28] with naphthoquinone nucleus is unknown. Threlfall et al (79) and Whistance and Threlfall (73) have shown that in maize shoots and Ivy leaves the nuclear methyl groups of vitamin K<sub>1</sub> are derived from the S. methyl group of methionine [29]. Further they have established that in maize shoots this methylation involves the transfer of an intact methyl group. A general outline of biosynthesis of vitamin K<sub>1</sub> [1] is given in scheme 10.

## 7. Pharmacology and Therapeutic Category

Phytonadione is a naturally occurring vitamin K which maintains a normal concentrations of prothrombin and other clotting factors in the blood plasma by promoting their biosynthesis in the liver (25,27,80). There is no generally accepted figures for human requirement of vitamin K, Frick et al (81) estimated the minimal daily requirement in vitamin K deficient patient as 0.03  $\mu$ g. In infant 10  $\mu$ g/kg of body weight is sufficient to prevent hypotherbinemia. The American Academy of Pediatrics Committee on Nutrition tentatively recommended that milk substitute formulas containing less than 25  $\mu$ g per litre of vitamin K should have phytonadione added to give a concentration of vitamin K not less than 100  $\mu$ g per litre (25,80,82).

Phytonadione is used to treat hypotherbinemia of various organs including those resulting from inadequate absorption of vitamin K or inadequate endogenous production of vitamin K (27,46,80,83-87), neonatal deficiency of prothrombin (88,89), and toxic action of salicylate, phenylbutazon and other toxic substances (46,90). It is also used in the treatment of severe haemorrhage due to low concentration prothrombin concentration caused by anticoagulant therapy with coumarin derivative such as Warfarin or indanedione derivatives such as phenindione (25,91-95). It is not effective in overdosage of with heparin. Phytonadione also have antiinflammatory effect (96). Only phytonadione should be used for the emergency treatment of drug-induced hypotherbinemia (97).

## 8. PHARMACOKINETICS

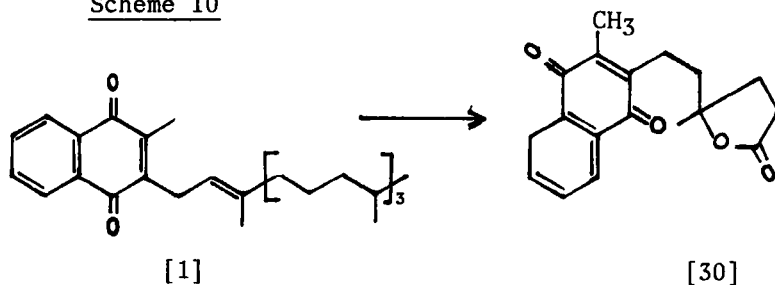
### 8.1 Absorption and distribution

Phytonadione is administered by oral, or Intra-venous or intra muscular routes. It is readily absorbed from the gastro-intestinal tract. Both bile and pancreatic lipase are necessary for the efficient absorption (25,98,99). After oral administration of the vitamin (10 mg) the maximum plasma concentrations occurred in about 3-4 hours (99,100). In infant after oral administration (20 mg) of phytamedione, the peak serum level occurs in 6-12 hours (100,101). After intra-venous injection of phytonadione (10 mg) the peak serum level occurs in 2-5 hours and the maximum values for prothrombin complex activity will be reached at 24-96 hours (102-104). Shorter duration of action was observed in cases of coumarin poisoning (104). It is not significantly stored by the body (25).

### 8.2 Metabolism

Phytonadione is metabolised to a  $\gamma$ -lactone [30] which is excreted as a conjugate glucouronide (98,99, 105-107) (scheme 11).

Scheme 10



### 8.3 Excretion

Phytonadione is excreted in as a conjugate in urine, faeces and bile (27,106).

### 8.4 Half-life

The average terminal half-life of phytonadione is about 1.7 hours (103,104).

## 9. MECHANISM OF ACTION

The best known function of phytonadione is to catalyse the synthesis of prothrombin by the liver. The vitamin K dependent step in clotting factor synthesis involves the post-ribosomal conversion of glutamyl residue into  $\gamma$ -carboxyglutamyl residues in clotting factors precursors. During the  $\gamma$ -carboxylation reaction phytonadione is converted into a biologically inactive metabolite, vitamin K<sub>1</sub> 2,3-epoxide. The epoxide is reduced back to the vitamin by a microsomal epoxide reductase and the cyclic interconversion of the vitamin and epoxide is referred to as the vitamin K<sub>1</sub>-epoxide cycle. The vitamin K-dependent carboxylase system was found in liver microsomes. A similar carboxylase system occurs in kidney, bone and probably in placenta and spleen (19,104,103-114).

## 10. TOXICITY

Excessive doses of phytonadione may produce haemolytic anaemia, hyperbilirubinemia and kernicterus in new born, particularly premature infants. Serious reactions following intraveineous use of phytonadione emulsion have been reported, these includes, flushing of face, sweating, cyanosis, a feeling of constriction of the chest and peripheral vascular collapse pain and nodules formation at the site of injuction may occur after intramuscular injection (27,46,115,116).

## 11. DOSAGE

As an antidote to anticoagulants, except heparin, 5 to 20 mg by mouth; 5 to 20 mg by intraveineously or intramuscularly, repeated in a few hours. In haemorrhagic disease of the new born: 0.5 to 1 mg intramuscularly or intraveineously (25,27).

## 12. PHARMACEUTICAL FORMS

1. Phytonadione Injections (USP 1975) (45)
2. Phytonadione tablet (USP 1975) (45)
3. Phytonadione capsule (B.P. 1968)(25)

### 13. METHODS OF ANALYSIS

#### 13.1 Elemental Composition

C=82.61%; H=10.29%; O=7.10% ( 24 )

#### 13.2 Identification

##### 13.2.1 Pharmacopeial tests

The following tests have been described in various Pharmacopoeiae for the identification of phytanodione

- a) Dam-Karrer reaction:-  
Dissolve 0.05 g of Phytonadione in 10 ml of methyl alcohol, add 1 ml of 20 per cent w/v sodium hydroxide in methanol, a green color is produced. Heat gently, the color changes to purple and then to reddish brown on standing (21,117).
- b) Dissolve 0.05 g of phytonadione in 10 ml of a mixture of methanol and ether (1:1), add a freshly prepared solution of 0.75 g of sodium hydrosulfite in 2 ml of warm water and shake vigorously a yellow color disappears immediately (26).
- c) The light absorption, in the range 230 to 350 nm, of a 2-cm layer of a 0.001 per cent w/v solution in trimethylpentane exhibits four maxima, at 243 nm, 249 nm, 261 nm, and 270 nm; extinction at 243 nm, about 0.80, at 249 nm, about 0.84, at 261 nm, about 0.77, and at 270 nm, about 0.78. The spectrum also exhibits minima at about 228 nm, 246 nm, 254 nm, and 266 nm. The ratio of the extinction at the minimum at about 254 nm to that at the maximum at about 249 nm is not less than 0.70 and not more than 0.75 (117).
- d) The light absorption, in the range 230 to 350 nm, of a 2-cm layer of a 0.01 per cent w/v solution in trimethylpentane exhibits a maximum at about 327 nm and a minimum at about 285 nm. The extinction at the maximum is about 1.4 and at the minimum about 0.44 (117).

### 13.2.2 Color tests

- a) Schilling - Dam test : Treatment of phytonadione with 5-imino-3-thioxo-1,2,4-dithiazolidine (xanthanehydride) and potassium hydroxide gives a stable orange color (118).
- b) Craven test : Treatment of phytonadione with alcoholic solution of ammoniacal cyanoacetic ester produces an intense blue colour (119).
- c) On mixing with sodium ethoxide phytonadione gives a blue color (120,121).
- d) When 2 ml of solution of phytonadione is treated with 2 drops of 10 percent hydroxylamine-hydrochloride and 1-2 drops of sodium hydroxide solution a red color is produced (122).
- e) Dissolve 0.15-0.5 mg of phytonadione in 0.5 ml of water. Add 0.1 ml of 1 percent solution of 2,4-dinitrophenyl hydrazine in 2 N hydrochloric acid, heat at 70° for 10 minutes, cool, add 0.3 ml of 20 percent sodium carbonate, add 1 ml of ammonium acetate and 1 ml of distilled water, a green color is produced (123).
- f) To 2 ml of an alcoholic solution of phytonadione, add 2 ml of 15 percent solution of sodium diethyl dithiocarbamate in alcoholic alkali, a Cobalt-blue color is produced which attains its highest intensity in 15 minutes and fades slowly after 8 minutes (124).

### 13.3 Titrimetric Methods

#### 13.3.1 Cerimetric

A cerimetric titration for the determination of the quinones including vitamin K was reported (125). The method is as follows:

2-80 mg of the sample is dissolved in 1-5 ml of 96 per cent ethanol, about 0.5-2 ml of stannic chloride in hydrochloric acid is added and the mixture is allowed to stand for 2 minutes. Then it is extracted with



chloroform. For each 0.5 ml of stannic chloride 1.5 to 2g of finely powdered potassium bicarbonate is added to the aqueous layer. After the gas development has ceased, about 5-6g of finely powdered anhydrous sodium sulphate is added. The mixture is shaken and filtered 10 ml of the filtrate is pipeted out into a mixture of 50 ml of 96 percent of ethanol and 30 ml of 10 percent sulphuric acid. Then 1 drop of the 0.2 percent alcoholic solution of *p*-ethoxy chrysoidine-hydrochloride is added and titrated against 0.05 N ceric sulphate till the red color changes to yellow.

### 13.3.2 Electrometric

#### 13.3.2.1 Polarographic

Hershberg et al (126) have polarographically determined phytonadione in aqueous isopropanol containing potassium chloride. The half-wave potential for reduction at the dropping mercury electrode is 0.58V. For quantitative polarographic estimation Knobloch (127) suggested a mixture of 0.06 N ammonium chloride in 75 percent isopropanol.

Burger (128) has reported a polarographic method for the determination of vitamins K. The sample containing 2-3 mg of vitamin K was rubbed with 3 ml of 0.5M potassium chloride solution. About 10 ml of the solution was polarographed from 0.2V. Maximum relative error was  $\pm 5$  percent. This method was useful for determination of vitamin K<sub>1</sub> in the presence of other vitamin. This method was extended to determine vitamin K in multivitamin preparations (129).

Hayakawa and Takamura (130,131) have described a polarographic method for consecutive determination of phytonadione and other K

vitamins. Phytonadione, menadione and menaphthone show well defined polarographic reduction waves in 80 percent ethanol solution containing acetate buffer and sodium chlorate. The total concentration of vitamins K is first obtained by measurement of the height of the reduction wave. By addition of an excess of cysteine to the solution the diffusion of vitamin K<sub>3</sub> disappears completely. Thus the concentration of vitamin K<sub>1</sub> can be determined.

Polarographic study of the reaction of K vitamins and thiols and consecutive determination of K vitamins was also reported by Takamura et al (132).

A differential pulse polarographic technique for the determination of vitamins including phytonadione in pharmaceutical preparations was reported by Lindquist and Farroha (133). The pulse technique gives lower detection limits, in the range 0.01 to 1 p.p.m. Vire and Patriarche (134) have described the behaviour of phytonadione and menaphthone in d.c., a.c. and differential pulse polarography. From 50 M to 0.5 mM-phytonadione can be determined at pH6 by d.c. polarography.

Heart et al (135) have determined the phytonadione in plasma by using differential pulse polarographic technique. The method is as follows: The phytonadione was extracted from the plasma with methanol-chloroform (2:1). The chloroform phase of the extract was evaporated and the residue was dissolved in ethanol - 0.5M-acetate buffer of pH 6.0 (9:1). Then phytonadione was determined by differential pulse polarography. The calibration graph was rectilinear in the range 80 to 1200 ngm<sup>-1</sup> and average recoveries were 72.2 per cent. The coefficient variation was 3.0% at a concentration of 2.75 gml<sup>-1</sup> of plasma.

### 13.3.2.2 Potentiometric

Karrer and Geiger (30 ) have reported the oxidation - reduction potential  $E_m = 0.005V$  for vitamin  $K_1$  in 80 per cent ethanol solution 0.02 N in acetic acid and 0.02 N in sodium acetate at 25°. Reigel et al (136) have found that oxidation - reduction potential of pure vitamin  $K_1$  in 95 percent ethanol, 0.2 N in hydrochloric acid and 0.2 N lithium chloride, was found to be 0.363V at 20°.

Trenner and Bacher (137) have described a potentiometric method where by vitamin  $K_1$  and other quinono-like substances whose standard oxidation - reduction potentials,  $E_0$  are less than about 0.5V. may be assayed. The procedure consists of two stages, first catalytic reduction of the quinoine to the hydroquinone and second the oxidation of an aliquot part with a relatively stable and easily obtained dye 2,6 dichlorophenolindophenol. This method is useful for determination of vitamin  $K_1$  in the presence of vegetable oils.

## 13.4 Spectroscopic Methods

### 13.4.1 Colorimetric

Based on the Dam-Karrer (21) reaction, Almquist and Klose (138) has developed a method for the determination of vitamin  $K_1$  in crude extracts. The color reaction is

carried out easily by dissolving a few milligrams of concentrate in 1 or 2 ml of methanol and then adding 1 ml of sodium methoxide. When warmed gently the mixture slowly develops a distinct purple color. The carotenoid pigments may be removed by partition with hydrocarbon solvent. The color due to reaction of the vitamin with sodium methoxide remains in the methanol phase. The vitamin is then assayed by measuring the extinction of the colored solution at 410 nm against reagent blank.

The stable orange color produced by the reaction of phytonadione with 5-Imino-3-thiono-1,2,4-dithiazolidine and potassium hydroxide (118) was applied by Schilling and Dam (139) to estimate phytonadione in plant materials. The procedure is as follows:

Extract the sample containing vitamin  $K_1$  with light petroleum and pass the extract through a column of activated calcium hydrogen orthophosphate. Elute with light petroleum until the  $\beta$ - and  $\alpha$ -carotene bands have passed through; chlorophylls and xanthophylls remain on the column. Evaporate the eluate to dryness at 20° under reduced pressure and dissolve the residue in 3 ml of benzene. Add 1.5 ml of an 80%-saturated ethanolic solution of 5-imino-1:2:4-dithiazolidine-3-thione and heat in a water bath at 50° for 5 min. with a 4 N solution of potassium hydroxide in 0.5 ml of methanol. Cool rapidly and extract with a mixture of light petroleum and 2 ml of 50% ethanol and then with 10 ml of light petroleum. Measure the extinction of the aqueous phase at 410 nm against a reagent blank. No loss of vitamin  $K_1$  was apparent in recovery experiments, and the content of

vitamin K<sub>1</sub> in a sample of lucerne meal estimated by this method was in good agreement with biological determinations.

A colorimetric oxidation - reduction method for the determination of the K vitamins was reported by Scudi and Buhs (140). The colorimetric method described is based on the oxidation - reduction titration reported by Trenner and Bacher (137). The vitamin is reduced in butanol solution in the presence of phenosafranine as the indicator. The resulting vitamin hydroquinone is then treated with an excess of a butanol solution of 2,6-dichloroindophenol in the absence of the air and the diminution of the color of the indophenol is a measure of the quinone originally present. As little as 5  $\gamma$  of vitamin K<sub>1</sub> per ml of the solution can be determined. The reduction of the vitamin quinone is completed within 3 minutes. The results are in essential agreement with bioassays and good recoveries of added vitamin K<sub>1</sub> can be obtained. Working under nitrogen at reduced pressure and in the absence of light gives better results. (141).

Ashbel *et al* (142) have described a new quantitative method for determination of vitamin K in blood serum. The method is as follows.

About 0.5 ml of blood serum is taken in each of four glass stoppered centrifuge tubes. One ml of 5 percent acetic acid is added to the first tube and no acid is added to the second tube. To the third tube 0.5 ml of water is added and the fourth tube contains 0.5 ml of standard solution of the vitamin and 1 ml of 5 percent acetic acid. To each tube 2 ml of aniline is added and the contents are mixed and heated on a water bath for 15 minutes at 90°. To the warm mixture 10-12 drops of toluene is added, mixed and centrifuged. The upper layer is separated and kept. The precipitate is washed with toluene and added to the 1st portion. The combined extracts are dried over anhydrous sodium sulphate and the extinction is then determined. The amount of the vitamin K present is calculated against the standard.

Vire and Patriarche (143) have reported a spectrophotometric method for the determination

of K vitamins. The procedure is as follows.

To a methanolic solution of the vitamin are added 14 M-sulphuric acid, 0.5 ml of 15 percent Titanium sulphate [ $\text{Ti}_2(\text{SO}_4)_3$ ] solution in 23 percent sulphuric acid and 0.3 ml of standard solution of Titanium chloride ( $\text{TiCl}_4$ ) in methanol. The mixture is rapidly cooled, diluted to 25 ml with 14M-sulphuric acid and kept in dark. The extinction is then measured between 640 and 655 nm for phytonadione. The calibration graphs are rectilinear for upto 0.4 to 4 mg  $\text{dl}^{-1}$  of phytonadione.

#### 13.4.2 UV Spectrophotometric

The British Pharmacopoea (117) described a UV spectroscopic method for the determination of phytonadione. The procedure is as follows:

Assay : Dissolve 0.1 g in sufficient trimethylpentane to produce 200 ml. Transfer 2 ml of this solution to the top of an alumina column prepared by adding 4.5 g of alumina (7 per cent water) to a glass tube of 5 mm internal diameter filled with trimethylpentane, allowing to settle, and reducing the depth of the layer of solvent above the alumina to about 2 mm. Carry out the chromatographic procedure with 20 ml of a mixture of one volume of anaesthetic ether and 49 volumes of trimethylpentane. Dilute the eluate to 50 ml with trimethylpentane and measure the extinction of the solution at the maximum at about 249 nm. Calculate the content of vitamin  $\text{K}_1$  taking 420 as the value of  $E(1 \text{ per cent, } 1 \text{ cm})$  at the maximum at about 249 nm.

The United States Pharmacopeia XIX (45) used a thin layer chromatographic purification of phytonadione before UV spectroscopic determination. The Assay procedure is as follows:

Divide the area of the Chromatographic Silica plate into three equal sections, the left and right sections to be used for the Assay preparation and the Standard preparation, respectively, and the center section for the blank. Apply 200  $\mu\text{l}$  each of the Assay preparation and the Standard preparation as streaks

2.5 cm from the bottom of the appropriate section of the plate. (In the period following the application of each preparation on the thin-layer plate and prior to the chromatographic development, protect the material on the plate from light, such as by covering the segments with strips of black paper, taking care to ensure that none of the material is removed from the plate.)

Develop the chromatogram in a suitable chamber, suitably protected from light, previously equilibrated with a 4:1 mixture of cyclohexane and ether and lined with absorbent paper, until the solvent front has moved 15 cm above the initial streaks. Remove the plate, allow the solvent to evaporate at room temperature, and locate the principal band occupied by the Standard preparation by viewing under short-wavelength ultraviolet light. Mark this band, as well as the corresponding bands in the Assay preparation and blank sections of the plate. Remove the silica gel from each band separately, either by scraping onto glazed weighing papers or by using a suitable vacuum collecting device, and transfer it to a glass-stoppered, 50-ml centrifuge tube. To each tube add 25.0 ml of dehydrated alcohol, insert the stopper, and shake by mechanical means for about 10 minutes. Centrifuge the tubes for 5 minutes. Concomitantly determine the absorbances of the solutions in 1-cm cells at the wavelength of maximum absorbance at about 248 nm, with a suitable spectrophotometer, using the solution from the blank preparation as the blank. Calculate the quantity, in mg, of  $C_{31}H_{46}O_2$  in the Phytonadione taken by the formula  $50C(Au/As)$ , in which C is the concentration, in mg per ml, of USP Phytonadione Reference Standard in the Standard preparation, and Au and As are the absorbances of the solutions from the Assay preparation and the Standard preparation, respectively.

The pharmacopeia of Japan (26) described a direct UV spectroscopic method for Assay of phytonadione.

Assay Weigh accurately about 0.1 g of Phytanadione, dissolve in sufficient isooctane to make exactly 100 ml. Measure accurately 10 ml of this solution and add sufficient isooctane to make exactly 100 ml. Pipet 10 ml of this solution and add sufficient isooctane to make exactly 100 ml. Read the absorbance  $A$  of this solution at the maximum wavelength, at about 248.5 nm, adjusting the slit of a spectrophotometer to band width of 0.5 nm (width at half-height). Complete the assay promptly and exercise care throughout the procedure to keep to a minimum the exposure to light.

$$\text{Amount (mg) of phytanadione} = \frac{A}{422} \times 100000$$

Chance et al (144) have reported a spectrophotometric method for the determination of K vitamins, ubiquinone etc. The extinction of the solutions containing the samples are measured between 200 and 300 nm.

Spectrophotometric determination of vitamins of K groups in concentrates and pharmaceutical preparations after chromatographic separation on silufol was reported by Rittich et al (145).

Vitamins  $K_1$ ,  $K_3$  and  $K_4$  (phylloquinone, menaphthone and acetomenaphthone, respectively) can be separated with use of benzene, chloroform or benzene - ethyl methyl ketone (3:1) as developing solvent and detected as their violet oxidation products by spraying with 70% perchloric acid and heating at 105° for 5 to 10 min. The application of this technique in combination with u.v. spectrophotometric determination of the vitamins has been investigated. Coefficients of variation of 1 to 2.5% have been obtained.

A UV spectroscopic method for the determination of vitamin  $K_1$  from alfalfa leaf was described by Tendille and Gervais (146). The method is as follows. The alfalfa leaf was extracted with acetone containing little water. The extract was then evaporated in vacuo. The residue was dissolved in isooctane and separated on a column of 2 g of silicic acid containing 5 percent water and 1 g of chromosorb W



by eluting first with isooctane and then with ether. The ether fraction contains vitamin K<sub>1</sub>. The fraction were identified by ultraviolet spectrophotometry. The solvent was removed and the residue was dissolved in ethanol and the extinction is measured at 270 nm.

#### 13.4.3 IR Spectrometry

Noll (147) has described a novel method of quantitative infra-red spectrometry as well as for the identification of vitamin K homologs. The vitamin K<sub>1</sub> are phytyl and the K<sub>2</sub> are polyisoprenoid, derivatives. Absorption bands near 1100 and 840 cm<sup>-1</sup>, present in the K<sub>2</sub> but not in the K<sub>1</sub> spectra, permit qualitative differentiation between the two homologous series. The length of the side-chain is determined within  $\pm 0.2$  isoprenoid unit by measurements of the relative intensities of certain bands. There is a linear relationship between the number of isoprenoid units in the side-chain and the ratio of the main alkyl stretching and bending bands to the extinctions of the characteristic naphthaquinone bands. These ratios are characteristic for each homologs. Independence of instrumental factors is obtained by a novel slit-width-calibration procedure with commercial DL-vitamin K<sub>1</sub> as the standard.

#### 13.4.4 Fluorimetric

Jansson (148) has studied the photoinduced fluorescence of phytonadione in solution. Phytonadione in petroleum ether or ethanol solution at room temperature was found to exhibit a photoinduced fluorescence when the solutions were irradiated with the 365 nm. mercury line. With prolonged irradiation the fluorescence intensity increased gradually to a maximum and then decreased. The intensity in petroleum ether solution was found to be directly proportional to the concentration of the phylloquinone in the range of 5-20  $\mu\text{g/ml}$ .

Aaron et al (149) have developed a quantitative photochemical-fluorimetric method for the determination of vitamin K<sub>1</sub>. The method

depends on measurement of the fluorescence of the product of ultraviolet irradiation of phytonadione solution in 1,4-dioxan with use of a 200-W Combined xenon-mercury lamp as excitation source and digital intergration or analogue recording of inluorescence at 431 nm. The detection limit is 5 p.p.b. The Fluorescence-spectroscopic technique was employed to determin phytonadione and other vitamains in biological samples, foods and drugs (150).

Fluorimetric determination of phytonadione and menatetrenone in biological materials by using high-performance liquid chromatography was described by Abe et al (151). Phytonadione was extracted from samples of plasma or liver with water-isopropyl alcohol mixture, reextracted with hexane and separated on a column of Nucleosil C<sub>18</sub> with ethanol-water (97:3) as a mobile phase. Then fluoremetric detection at 430 nm (excitation at 320 nm) was used after reaction of eluted compounds with ethanolic 0.08 percent sodium borohydride in a reaction cell. Recoveries of 100-ng amount of phytonadione added to samples were 96 to 100 percent. The limit of detection for each compound was 0.4 ng. The coefficient of variation was typically 2 percent. Method is extended to determine the phytonadione epoxide in biological materials (152).

A improved method of HPLC combined with fluorimetric detection was applied by Langenberg and Tjaden (153) to the study of phytonadione and phytonadione epoxide in plasma samples.

Holasova and Blatna (154) developed a fluorimetric method for the determination of phytonadione. The method is as follows.

Ethanolic solution of phytonadione (0.01 to 10  $\mu\text{g l}^{-1}$ ) was irradiated at 366 nm for 15 minutes and the photo induced fluorescence was then measured at 405 nm (excitation a 330 nm). The concentration of phytonadione was found from a calibration graph, which was rectilinear for 0.01 to 3  $\mu\text{g l}^{-1}$ . The coefficient variation was 3.54 percent.

### 13.5 Chromatographic Methods

#### 13.5.1 Column Chromatography

Column chromatographic (CC) method has been extensively used for the separation and purification of vitamin K<sub>1</sub>.

Dam and Schonheyder (17) separated vitamin K by adsorption on calcium carbonate or sucrose. Karrer et al (31) have isolated K vitamins from alfalfa after chromatography on Magnesium sulphate, zinc sulphate and zinc carbonate.

Mckee et al (22) and Binkley et al (56) used primarily chromatographic adsorption on Decalso and Permutit on a large scale in their isolation of the vitamin from a petroleum ether extract of alfalfa meal. Dam and Glavind (155) have described the method of purification of K vitamins on calcium sulphate, Barium sulphate and Alumina. Egger (57) used silica gel for column chromatographic separation of vitamin K<sub>1</sub> from the plant materials.

Mayer et al (43) isolated vitamin K<sub>1</sub> from alfalfa meal applying an efficient method of purification by partition chromatography on polyethylene powder in a reversed phase methanol-water-hexane system.

A reversed-phase partition column chromatography method was developed by Losito and Millar (156) for preparative separation of vitamin K<sub>1</sub> and K<sub>3</sub> from other K vitamins. A silicic acid column was prepared and the vitamins were separated by reversed phase partition on ether-n-hexane system in the order of K<sub>1</sub> 0.5%; K<sub>2</sub> and K<sub>3</sub> 1.07; K<sub>3</sub>, 4.0%.

Matschiner and Taggart (157) separated vitamin K and associated lipids by reverse-phase column chromatography. The column described was packed with hydrophobic Celite-polyethylene powder (1:1). The stationary phase was hexane or 2,2,4-trimethylpentane and the developing solvent is isopropyl alcohol-acetic acid and water in various proportions. By this method effective separation was achieved.

Williams (158) used a column chromatographic method for the separation and determination of vitamin K<sub>1</sub>, other quinones and -tocopherol from plant materials. The method is as follows:

Freeze about 30 g of the vicia faba leaves in liquid air and grind with sodium disodiumhydrogen phosphate, lyophilize over night at 4 to 6°. in the dark. Grind the materials again, extract with acetone and evaporate to dryness. Dissolve the residue in hexane. Pass the solution through a column packed with Kiesel G-Celite (1:1) Elute consecutively 60 ml each of 0.5, 3, and 10 percent and finally 20 percent ether in hexane. Vitamin K<sub>1</sub> is then determined from the solution by UV spectrometry.

#### 13.5.2 Paper Chromatography

Green (159) has described a paper chromatographic method for the detection of vitamin K<sub>1</sub>. About 10 g of vitamin K<sub>1</sub> was chromatographed on Whatman No.1 filter paper impregnated with silicone with 75 percent (v/v) aqueous ethanol as a solvent. When the paper was dried and held before ultraviolet lamp (3665A.), an area of red fluorescence was seen at R<sub>f</sub> 0.20.

Green and Dam (160) have studied in detail about the paper chromatography of vitamin K and related compounds on siliconised paper. Reversed-phase chromatography was applied. The R<sub>f</sub> values of vitamin K<sub>1</sub> in different solvent systems like ethanol-acetic acid-water; isopropanol-acetic acid-water and n-propanol-acetic acid-water were 0.28, 0.27 and 0.36 respectively. The exposure of the spots to U.V. lamp for 45 to 65 seconds induced a permanent change in the fluorescent color. Spraying with ethanolic potassium hydroxide after activation produced a stable green color. The sensitivity of this method was 0.5 µg for qualitative and 1.5 µg for quantitative analysis.

Lester and Ramasarma (161) have separated vitamin K<sub>1</sub>, vitamin K<sub>2</sub> and vitamin E on silicone-impregnated paper with n-propanol-water (7:3 or 3:1) Zinc carbonate treated paper was used in two-dimensional chromatography for the separation of vitamin K<sub>1</sub> and coenzyme Q compounds (162).

Blattna et al (163) have used centrifugal chromatography for the separation of vitamin K<sub>1</sub> and other fat soluble vitamin. The centrifugal apparatus of Pavlich et al (164) was used. The chromatographic system consisted of 20 centimeter diameter whatman paper No.3, impregnated with a 10-20% paraffin oil in petroleum ether solution as a stationary phase and anhydrous methanol or ethanol as a mobile phase. The solvent was applied at 0.7-1.5 ml per minute with a centrifugal spinning at 500 r.p.m. Vitamin K<sub>1</sub>, K<sub>2</sub> and K<sub>3</sub> were well separated in anhydrous methanol. For simultaneous determination of fat soluble vitamins including vitamin K<sub>1</sub> by circular paper chromatography using butanol - acetone - ethanol-water system was described by Barbiroli (165).

Green et al (166) used paper chromatography for studying the chemical structure of vitamins K and other related compounds. Whatman No.4 paper impregnated with 5 percent olive oil in ether or paraffin in light petroleum was used as a stationary phase and 50-95 percent ethanol was used as a mobile phase.

Lichtenthaler (167) has separated K vitamins and other fat soluble vitamins from the biological materials by chromatography in the dark on filter-paper impregnated with alumina filler. Benzene or benzene chloroform (1:1), cyclohexane or cyclohexane-benzene (3:7) were used as solvents. The chromatograms were examined in day light and in UV light; in antimony trichloride solution or 2,2'-bipyridyl-ferric chloride solution and neotetrazolium reagent.

### 13.5.3 Thin-Layer Chromatography

Katsui et al (168) and Ishikawa and Katsui (169) employed thin-layer chromatographic technique for the detection of vitamins K and other fat soluble vitamins. Kiesegel G, aluminium oxide G were used as adsorbent; chloroform and benzene were used as suitable solvents. 60 percent perchloric acid, 95 percent sulphuric acid and antimony trichloride were used as detecting reagents.

Egger (57) separated vitamins K from the lipids of leaves using column and thin layer chromatography and quantified the vitamin content by visually comparing the unknown with the known standards of authentic quinones. Egger and Kleinig (170) separated K vitamins by using a thin layer chromatographic technique on polyamide with methanol-methyl ethyl ketone-water solvent system.

Matschiner and Taggart (157) combined reverse phase column chromatography and paraffin impregnated TLC technique to resolve vitamin K and qualitatively assessed the various forms of vitamin K in liver and organ extracts.

Dummler and Dorfling (171) separated vitamin K<sub>1</sub> and other quinones by using TLC technique.

A good separation of vitamin K<sub>1</sub>, vitamin K<sub>2</sub> and vitamin K<sub>3</sub> was obtained on a kieselgel G-Kieselgur G (1:1) plates impregnated with paraffin. The plate was developed in acetone-water (9:1) for 30 minutes and the spots were revealed with 0.05 percent Rhodamine B solution.

The TLC of vitamin K<sub>1</sub> and other fat soluble vitamins on aluminium oxide layer are extensively studied by Davidek (172) and Blattna and Davidek (173). The different solvent systems and the R<sub>f</sub> values are tabulated (Table 5).

TABLE 5

Solvent System	R <sub>f</sub> values	Color of the spots	
		70% perchloric acid	98% sulphuric acid
Methanol	0.78	yellowish brown	yellowish brown
Anhydrous ethanol	0.89	"	"
n-Butanol	0.92	"	"
Benzyl alcohol	0.89	"	"
Hexane	0.85	"	"
Cyclohexane	0.90	"	"
Petroleum ether	0.31	"	"
Petrol	0.21	"	"
Benzene	0.94	"	"
Toluene	0.90	"	"
Xylene	0.91	"	"
Chloroform	0.94	"	"
Carbon tetrachloride	0.74	"	"

Hashmi et al (174) have developed a circular thin-layer chromatographic method for semi-quantitative determination of fat-soluble vitamins including vitamin K<sub>1</sub>. This technique involves the development of rings on chromatoplates of either alumina or silica gel with circular TLC apparatus. Cyclohexan-ethyl ether was used as a developing solvent. Concentrated sulphuric acid, saturated solution of anti-money pentachloride in carbontetrachloride and 70 percent perchloric acid were used as a spray reagent. The R<sub>f</sub> value of vitamin K<sub>1</sub> and maximum sensitivity standard (in µg) on aluminium-oxide and silica gel are 0.85; 0.7; 0.84, 1.0 respectively. This method is applicable to determine vitamin K<sub>1</sub> in multi vitamins tablets.

Perisic-Janfic et al (175) have evaluated the various TLC materials for use in quantitative analysis of fat soluble vitamins including vitamin K<sub>1</sub> and found that silica gel G and alumina are the best adsorbent for the separation of these vitamins.

The applications of densitometry in thin-layer chromatographic determination of vitamin K<sub>1</sub> was reported by Manes et al (176). At first the vitamin K<sub>1</sub> is separated from the lipids by preperative column chromatography on neutral alumina. The eluate of the column is then chromatographed on adsorbosil 5 prekote layer with a standard solution of vitamin K<sub>1</sub>. The chromatogram was first developed in carbontetrachloride and then in benzene. After the benzene had volatilized, each layer of the chromatogram was scanned with a chromatogram spectrophotometer. The adsorption spectra and the standard vitamin K<sub>1</sub> was determined by reflectance densitometry. The area of the vitamin K<sub>1</sub> peak was calculated by triangulation and a standard curve was plotted as a log of the weight of the standard vitamin K<sub>1</sub> against the square route of the area under the curve obtained by densitometry. The amount of vitamin K<sub>1</sub> in a sample was determined by comparison with standard curve.

#### 13.5.4 Gas Liquid Chromatography

A GLC Method for the identification of phytonadione has been carried out in our

laboratory using a varian GC-3700 gas chromatograph equipped with Varian CDS 111 integrator.

Column condition: 3% OVI on Gas chrom Q (80-100 mesh); glass column (2m x 2mm). The column run at 260°-300°C. The temperature was increased by 10° per minute.

Carrier gas: Nitrogen, flow rate was adjusted to 50 ml per minute. Detector: FID at 300°C hydrogen and air flow rate were adjusted to 30 ml per minute and 300 ml per minute respectively. TriSil was used as a solvent. Sample size was 2 µl. The injection temperature was 250°C and the chart speed was adjusted to give 0.5 cm per minute. The retention time = 6.4 minutes. The GLC of phytonadione is presented in Fig. 7.

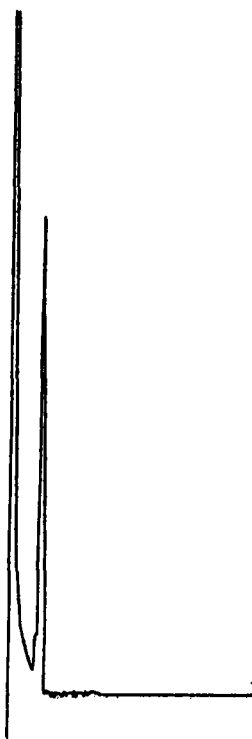


Fig. 7: GLC of Phytonadione



GLC has been used to quantitate the various forms of vitamin K. The gas chromatographic behaviour of vitamin K<sub>1</sub> has been studied by Nair and Turner (177), Carroll and Herting (178). Ninomiya *et al* (179) and Dialameh and Olson (180). Libby *et al* (181) have reported a GLC method for determination of vitamin K<sub>1</sub>. A gas chromatograph equipped with hydrogen FID was used. [The column condition is presented in table 6]. By using reference standard solution of vitamin K<sub>1</sub> (10 µg/ml) a calibration curve was prepared. A measured volume of assay solution in n-hexane was injected, the area of the peak was measured and the amount of vitamin was found by reference to the calibration curve.

Sheppard (182) determined vitamin K<sub>1</sub> in biological fluid by using GLC technique. The column condition is shown in table 6.

Vecchi *et al* (183) have described a GLC method for the determination of the diastereomers of vitamin K<sub>1</sub>. The diastereomer of vitamin K<sub>1</sub> was determined as their dihydrodimethyl ether by GLC on glass column coated with Silar 10C. With hydrogen as carrier gas and FID. This method in conjunction with optical rotation measurement are useful for characterisation of phytonadione samples. Electron-capture gas-liquid capillary chromatography was employed by Bechtold *et al* (184) for quantitative analysis of vitamin K<sub>1</sub> and vitamin K<sub>1</sub> 2,3-epoxide in human plasma. By using a fused-silica column coated with CP Sil 5CB, complete base line separation of the two compounds was achieved.

Many other GLC methods (185-189) for the determination of phytonadione have also been reported (Table 6).

### 13.5.5 High Performance Liquid Chromatography

High-performance liquid chromatography was employed for the separation of phytonadione and related compounds (190). The compounds are separated by HPLC on Bondapak C<sub>18</sub> with methanol as a mobile phase.

Yamano et al (191) have studied the photo isomerisation of phytonadione and menaquinone-4 in solutions for injection or infusion by HPLC. The *cis*- and *trans*-isomers of the cited compounds have been separated on a column (25 cm x 4.6 mm) of Nucleosil 50 with hexane-dibutyle ether (47:3) as a mobile phase (1.5 ml/min) and detection at 254 nm.

Resolution of phytonadione, phytonadione 2,3-epoxide, 2-chlorophytonadione and their geometric isomers by HPLC was reported by Haroon et al (192). Separation of the *cis*- and *trans*-isomers of these compounds by adsorption HPLC was achieved on a column (25 cm x 5 mm) of Spherisorb-5 with hexane-dichloromethane (3:1) as a mobile phase and 250 nm detection.

Prenzel and Lichtenthaler (193) described a HPLC method for separation of phytonadione and other prenylquinones. The cited classes of the compounds were separated by HPLC on column (25 or 12.5 cm x 3 mm) of Lichrosorb-si 60 plus Lichrosorb-diol or of Lichrosorb RP-8 with dioxane in hexane and aqueous methanol respectively.

Multi-dimensional HPLC for the determination of *trans*-phytonadione was described by Lefevre et al (194). The method is as follows:

*Trans*-phytonadione is extracted from deproteinised blood serum with hexane and separated from most of the co-extracted lipids by adsorption chromatography on column (20 cm x 7 mm) of totally porous Alltech Rsil with a guard column (10 cm x 4.6 mm) of Perisorb A and 3% of isopropyl ether in hexane as a mobile phase. *Trans*-phytonadione is further separated from co-eluted compounds, and determined on a column (15 cm x 3.2 mm) of RP-18 with ethanol as mobile

Table 6 : GLC condition used for phytonadione

S.No.	Column	Support	Liquid phase	Carrier gas	Flow rate	Internal standard	Temperature	Detector	Ref.
1	183 cm x 5mm	Gas-chrom P 60/80 mesh	SE 52	Argon	75ml/min	-	228°C	Argon ionization detector	177
2	Pyrex 6 feet x 4mm	Gas-chrom P 100/120 mesh	1% SE-30	Hydrogen	40ml/min	-	250°C	FID	181
3 508	Pyrex 6 feet x 3 mm	Gas-chrom P (silainised) 100/120 mesh	1% SE-30	Argon Hydrogen	100ml/min	-	230°	Argon ionization detector or hydrogen fame ionization	182
4	6 ft x 0.125 in	Gas-chrom P siliconised 100/140 mesh	3% SE-30 or Qf-1-0065	-	-	-	205°C or 195°C	-	178
5	2M x 2.3 mm	Gas-chrom Z 80/100 mesh	A piezonN	Helium	40ml/min.	octa-cosane	250°	FID	185
6	Glass 120cm x 6mm	Celite 545 100/120 mesh	10% A piezon L or silicon - elastomer SE 30	Argon	70ml/min	-	150-200°	Argon ionization detector	186

S.No.	Column	Support	Liquid phase	Carrier gas	Flow rate	Internal standard	Temperature	Detector	Ref.
7	Glass 122cm x 2mm	Diatoport S	3.8% SE	Helium	25ml/min	-	235°-295°	H. flame ionization detector	180
8	Glass 2m x 3mm	Chromsorb W AWD MCS 60/80 mesh	3% OV-I	Nitrogen	60ml/min	triacon- tane	260°	H. Flame ionisation detector	187
9		Cromosorb G	2.5%Dexsil 300GC	Nitrogen	40ml/min	dotria contane	290°	FID	188
10	190cm x 2.2 mm	AnakromQ 90/100 mesh	3% OV-17	Nitrogen	80ml/min		302°	-	189

Table 7 : HPLC conditions for phytonadione

S. No.	Column	Support	Mobile phase	Flow rate	Internal Standard	Detector	Ref.
1	-	Nucleosil C <sub>18</sub>	Ethanol-methanol (3:2) containing 70% perchloric acid	-	-	Electro chemical	196
2	25cmX4.6mm	Nucleosil C <sub>18</sub>	Methanol-ethanol (7:3)	0.7ml/min	-	Fluorimetric	197
3	-	ZorbaxSI1	hexane-isoprophl ether (7:1) mixed with methanol-ethanol 7:1	-	-	Electro chemical	198
4	20cmX4.6mm	PerisorbA	hexane	-	phytonadione	UV	199
5	15cmX3.2mm	Rsil C <sub>18</sub> LL	methanol	1ml/min.	-	Fluorescence	200
6	30cmX3.9mm or 10cmX4.9mm or 25cmX4.6mm	Bondapak ODS or Lichrosorb 10 RP-8 or ZorbaxODS	Methanol water dichloromethane proportion	1ml/min.	-	UV	201

S. No.	Column	Support	Mobile phase	Flow rate	Internal Standard	Detector	Ref.
7	15cmX4.6mm	Nucleosil C <sub>18</sub>	acetonitrile propan-2-ol (9:1) containing 0.1% perchloric acid	-	-	Fluorescence	202
8	25cmX4.5mm	Ultrasphere ODS or partisil-10	Dichloromethane -acetonitrile (3.7) 20% acetonitrile in hexane	1ml/min.	menaquinone	UV	203
9	5cmX4.6mm	SHIM-PACK PC18-03/S0505	Methanol			UV	204

phase and detection at 248 nm. [ $^3\text{H}$ ] *trans*-phytonadione is used as a internal standard.

Haroon and Hauschka (195) applied the multi-dimensional HPLC technique to assay phytonadione in rat liver. The phytonadione was extracted from the homogenised liver tissue and filtered through glass microfibre. The filtrate was evaporated and the residue was dissolved in hexane and chromatographed on a column of Sep-Pak silica with ether-hexane. The elute was evaporated, the residue was dissolved in hexane and then applied to a column of Partisil 5 for HPLC with hexane - dichloromethan (half saturated with water) as mobile phase. The final separation was on a column of Zorbax ODS or Hypersil ODS with methanol-dichloromethan as mobile phase and detection at 270 nm.

Other HPLC methods for the separations and determination of phytonadione in biological fluids, liver, plasma and serum have been reported (196-204). The chromatographic conditions used for the analysis of the phytonadione is listed in table 7.

#### 13.5.6 Reversed-phase High Performance Liquid Chromatography

Zonta et al (205) have described a reversed-phase HPLC method for the separation and identification of phytonadione and other fat-soluble vitamins. The solutions containing the samples were analysed by reversed-phase HPLC on two columns in series, one (25 cm x 2.6 mm) of ODS-HC sil-x-1 a second (15 cm x 4.6 mm) of Supelcosil LC 18. The columns were protected by guard column of 40-  $\mu\text{m}$  pellicular LC18 and were operated at 44°; with mobile phase of a gradient of 15% water in acetonitrile-methanol (4:1).

Reversed-phased HPLC has been used extensively for the determination of phytonadione in food, pharmaceutical formulae and biological fluids. Other reversed-phase HPLC

Table 8 Reversed-Phase HPLC conditions for Phytonadione

S. No.	Column	Support	Mobile-phase	Flow rate	Internal standard	Detector	Ref.
1	25cm x 2.2mm	Varian Micro Park CH 10	Acetonitrile-water (99:1)	90 ml/h	-	UV	206
2	25cm x 4.6mm	Zorbax ODS	gradient elution of methanol-ethyl acetate (43:7) and 100% acetonitrile	-	cholesteryl phenylacetate	UV	207
3	15cm x 3.2mm	RP-18 LL	8% Dichloromethane in acetonitrile	-	-	UV	208
4	25cm x 4.6mm	Zorbax-ODS	Acetonitrile-Dichloromethane (7:3)	0.8 ml/min	-	UV	192
5	-	Hypersil-MOS	92.5% aqueous methanol	-	-	Fluorescence	209
6	25cm x 4mm	Bondapak C <sub>18</sub>	Methanol-acetonitrile-tetrahydrofuran-water (39:89:16:6)	1.5ml/min	-	UV	210
7	-	Spherisorb 5 ODS	Methanol	-	-	UV	211
8	-	Nova-Pak C <sub>18</sub> Radial-Pak L	gradient elution of ethanol water (9:1) - ethanol-hexane (9:1)	1.5ml/min	-	UV	212



methods are reported for separation and identification of phytonadione in various pharmaceutical forms, food and biological fluids (206-212). Table 8 shows various systems and conditions that have been used for the analysis of phytonadione.

### 13.6 Bio-Assay Methods

Most conveniently biological assay is carried out in chicks. The bio-assay is based on the measurement of clotting time of whole blood or measurement of prothrombin level in the blood. If sufficiently large number of chicks are included in each groups; the reciprocal of the mean clotting time and of the mean prothrombin time bears a linear relation to the log of the vitamin,  $K_1$  in the diet (213-215). Prothrombin has been determined by an elaborate two stage method (216, 217) or by a method (18) in which the ratio of the strength of a clotting agent (thromboplastin required to clot the plasma in a specific time to the strength required to clot normal plasma is determined. Several procedures have been reported (218-222).

Almquist and Stokstad (223 ) have described an assay procedure for the determination of antihemorrhagic vitamins. The assay is based on the blood clotting time. The assay procedure is as follows:

A group of newly hatched chicks are fed with vitamin K free diet for 3 to 4 days. This chicks are divided into two batches. One batch is fed with test feed and other is the control which is fed with diet containing the alfalfa extract equivalent to 1% as a standard. The bloods are drawn from the main wing vein and about 2 ml is placed in a clean glass vial held in a water thermostat at 38.5° and shaken mechanically by a constant speed mechanism. When the blood no longer flow it is considered to have clotted. The clotting time which is the time between sample withdrawal and time of clotting is noted. If the time exceeds 30 minutes, the sample is considered negative.

Matschiner and Doisy (224) reported a bioassay of antihemorrhagic vitamins in chicks based on the analysis of chicks blood prothrombin level. The assay procedure is as follows.

One day old chicks are placed in wire floor brooders and are fed for 10 days with a diet containing soya protein, glucose, corn oil, cellulose, a supplement of amino acid, minerals and vitamins (no vitamin K). After a initial period of growth and depletion of vitamin K, they are separated into a groups of 10 and fed for 4 days with diets containing materials to be assayed. The blood sample are taken simply either by cutting of the head of the chicks or from carotid artery or from jugular vein or from wing veins. Dilution of the normal chick plasma are clotted by the following additions: Russell's viper venom in cephalin suspension, beef plasma previously treated with barium sulphate and an aqueous solution of 35 m M calcium chloride. The prothrombin time in the blood sample is determined with the help of the clotting agent. The reciprocal prothrombin time bears a linear relation to log of vitamin K level in the diet.

Antidicumarol activity of vitamins K can be utilized for the bioassay of vitamin K<sub>1</sub> and other vitamins K. Such trials have been reported by several investigators in dogs, in rabbits, in rats, mouse and in men (225-230).

Lowenthal and Taylor (231) have developed a method for measuring activity of vitamin K<sub>1</sub> and other anti-hemorrhagic compounds in rats. Male rats weighing 175 to 225 g were given 0.5 g of 3-( $\alpha$ -acetylbenzyl)-4-hydroxy-coumarin, mixed with 5 g of animal feed for two days. The substances to be tested was injected intramuscularly 17 to 20 hours after the second dose of anticoagulant and its effect on blood clotting time was determined.

Tanabe (232) has described a bio assay of vitamin K<sub>1</sub> and other vitamins K preparations by means of antidicumarol activity measured by Thrombotest in mice. The method is as follows:

Three groups each consisting 10-15, mice weighing about 20-70 g are selected. As a dicumarol preparation, Warfarin was administered subcutaneously 16 hours prior to the test. To the another group of mice vitamin K<sub>1</sub> and warfarin were administered simultaneously. The third group (control) were treated neither with warfarin nor with vitamin K<sub>1</sub>. After 16 hours, the blood was taken from the heart exposed directly after being killed by a head blow.

About 0.5 ml of the blood was pipetted and treated with sodium citrate solution. Then the blood was treated with Thrombotest and the clotting time was measured.

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## PRALIDOXIME CHLORIDE

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## 1. INTRODUCTION

Pralidoxime chloride, a quaternary ammonium oxime is known to be a potent reactivator of cholinesterase which has been inhibited by certain organophosphorus compounds. The oxime, therefore, has been widely accepted as part of the therapeutic armamentarium against poisoning by these compounds.

Cholinesterase reactivation produced by pralidoxime occurs principally at the neuromuscular junction and results in reversal of anticholinesterase-induced paralysis of respiratory and other skeletal muscles. The drug also reactivates cholinesterase at autonomic effector sites and, to a lesser degree, within the CNS.

## 2. CHEMISTRY

- 2.1. Chemical Name and CAS Registry Number:  
2-Hydroxyiminomethyl-1-methylpyridinium chloride;  
CAS-51-15-0.
- 2.2. Synonyms:  
2-PAM chloride  
2-PAM Cl  
2-Pyridine aldoxime methochloride  
Pyraloxime chloride  
2-formyl-1-methylpyridinium chloride oxime  
1-methyl pyridinium-2-aldoxime chloride  
1-methyl-2-formylpyridinium chloride oxime  
N-methyl pyridinium-2-aldoxime chloride  
2-pyridine aldoxime methylchloride  
Protopam chloride

2.3. Chemical Formula:  $C_7H_9ClN_2O$

2.3.1. Elemental content:

C	48.71%
Cl	20.54%
N	16.23%
O	9.27%
H	5.25%

(Kondritzer, et al., 1961; %Merck Index, 1983).

2.4. Structural Formula:

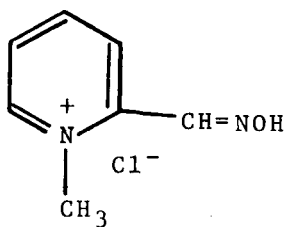


Fig. 1: Pralidoxime Chloride

2.5. Molecular Weight: 172.63

2.6. Physical Properties and Constants:

2.6.1. Nature: Crystalline, odorless, stable in air at temperatures below 100° C.

2.6.2. Color: White to pale yellow.

2.6.3. Melting point: 233-238°C with decomposition (Merck Index, 1983), 215-225°C with decomposition (Remington's Pharmaceutical Sciences, 1985).

2.6.4. pKa:

7.8 - Oxime group (Chemical Stability of Pharmaceuticals, 1986).

7.8 - 8 - Pralidoxime (AHFS, 1987)

2.6.5. Solubility: The solubility, g/100 mL of pralidoxime chloride in various solvents at 0 and 25° C is provided in Table-I (US Patent, 1964; Martindale, 1982).

TABLE I

## Solubility of Pralidoxime Chloride in Various Solvents

Solvent	Solubility (g/100 mL)	
	0°C	25°C
Acetone	<0.002	0
Isopropanol	0.053	0.09
Ethanol	0.435	0.89
Methanol	4.4	8.5
Water	54.3	65.5
Isopropanol, 10 volumes/ water, 1 volume	1.0	1.8

## 2.6.6. Absorbance Maxima (UV, IR):

In acidic solution, pralidoxime chloride exhibits an absorption maxima at 292 nm (Ellin and Kondritzer, 1959) and in basic solution, pralidoxime chloride exhibits significant bathochromic shift resulting in an absorption maxima at 335 nm which is attributed to the oximate anion (Ellin and Kondritzer, 1959; Groff and Ellin, 1969). The observed absorptivities and other spectrophotometric constants for pralidoxime chloride in various media are listed in Table II.

TABLE II

## UV Absorption Maximas and Minimas for Pralidoxime Chloride\*

Medium	$\lambda$ , nm		E	Fig. #
	Max.	Min.		
0.1 N HCl	292-29	-	12,400	2
	-	262	3,680	
0.1 N NaOH	335	-	18,700	3
		246	650	

Data adopted from Lehman and Bloch, 1965; May *et al.*, 1965.

\*Isosbestic point: UV absorption curves for pralidoxime chloride at various hydrogen-ion activities indicate that the isosbestic point of the oxime is at 307 nm.

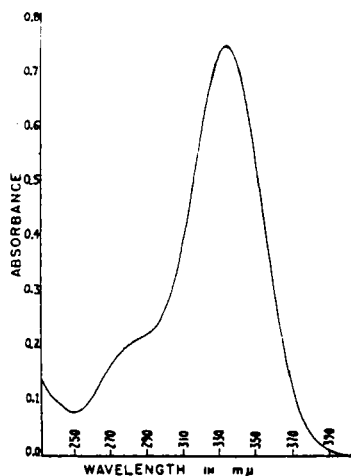


Fig. 2: Ultraviolet Absorption Spectrum of Pralidoxime Chloride in 0.1 N HCl

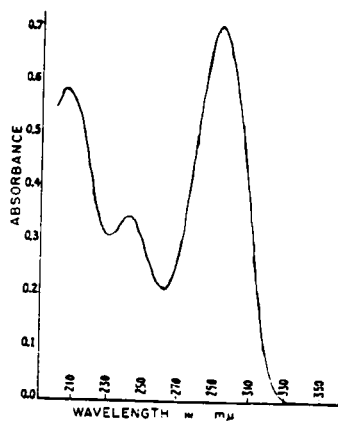


Fig. 3: Ultraviolet Absorption Spectrum of Pralidoxime Chloride in 0.1 N NaOH

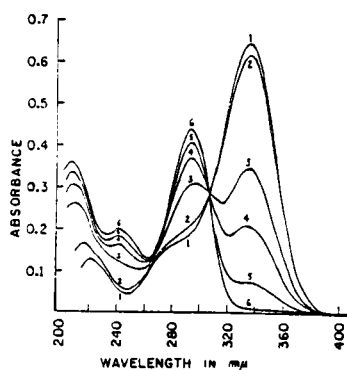


Fig. 4: Ultraviolet Absorption Spectra of Pralidoxime Chloride at various Hydrogen-ion Activities (1, pH 10; 2, pH 9.1; 3, pH 8; 4, pH 7.6; 5, pH 7; 6, pH 6.

The UV absorption spectra of pralidoxime chloride at various hydrogen-ion activities is provided in Fig. 4.

The infrared (IR) absorption spectra for pralidoxime chloride in a potassium bromide (KBr) disk and in a split mull are presented in Figs. 5 and 6, respectively.

#### 2.6.9. NMR and Mass Spectrometry:

The NMR spectrum for pralidoxime chloride is provided in Fig. 7 and the NMR for the iodide salt along with its corresponding assignments is presented in Fig. 8. Although, the mass spectrum for pralidoxime chloride is difficult to retrieve, the mass spectra of 1-methylpyridinium chloride which forms the primary nucleus for pralidoxime salts, is presented in Fig. 9.

#### 2.6.8. Neutralization Equivalent:

The neutralization equivalent of pralidoxime chloride ranges within a domain of 167-178 when determined by employing nonaqueous titration (May *et al.*, 1965).

Accurately weighed samples of pralidoxime chloride (50-100 mg) are dissolved in 40 mL of glacial acetic acid with slight warming. To this solution, 15 mL of a 5% solution of mercuric acetate in glacial acetic acid and 3 drops of a 1% solution of crystal violet indicator in glacial acetic acid are added. This solution is titrated against 0.1 N perchloric acid solution to an emerald-green endpoint. A reagent blank solution is titrated to the endpoint color and the blank value is subtracted from the sample value.

#### 2.6.9. Titration Curve:

The titration curve, as depicted in Fig. 10, for the neutralization of pralidoxime chloride is obtained by employing the following procedure. An accurately weighed sample of the oxime salt (~200 mg) is dissolved in 10 mL of carbondioxide-free, triple-distilled water. An electrode probe assembly is immersed in the solution; the surface of the solution is continuously flushed with nitrogen gas. The solution is titrated with 1.0 N NaOH solution from a microburette, the tip of which is kept beneath the surface of the solution. The hydrogen-ion activities are measured employing a pH meter.



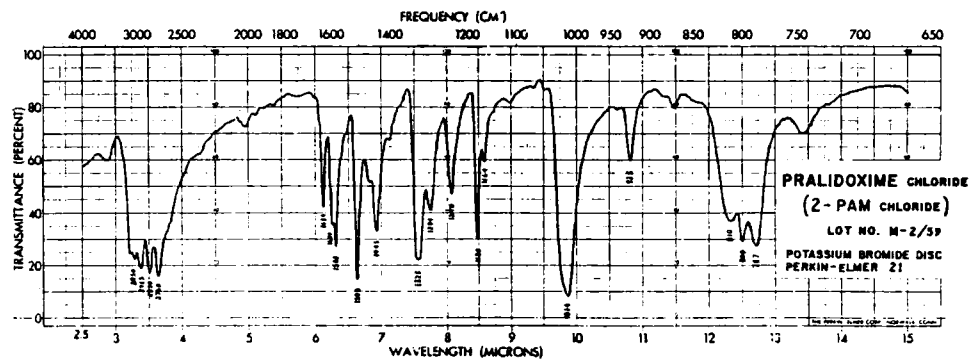


Fig. 5: Infrared Absorption Spectrum of Pralidoxime Chloride in Potassium Bromide Disk (0.4%)

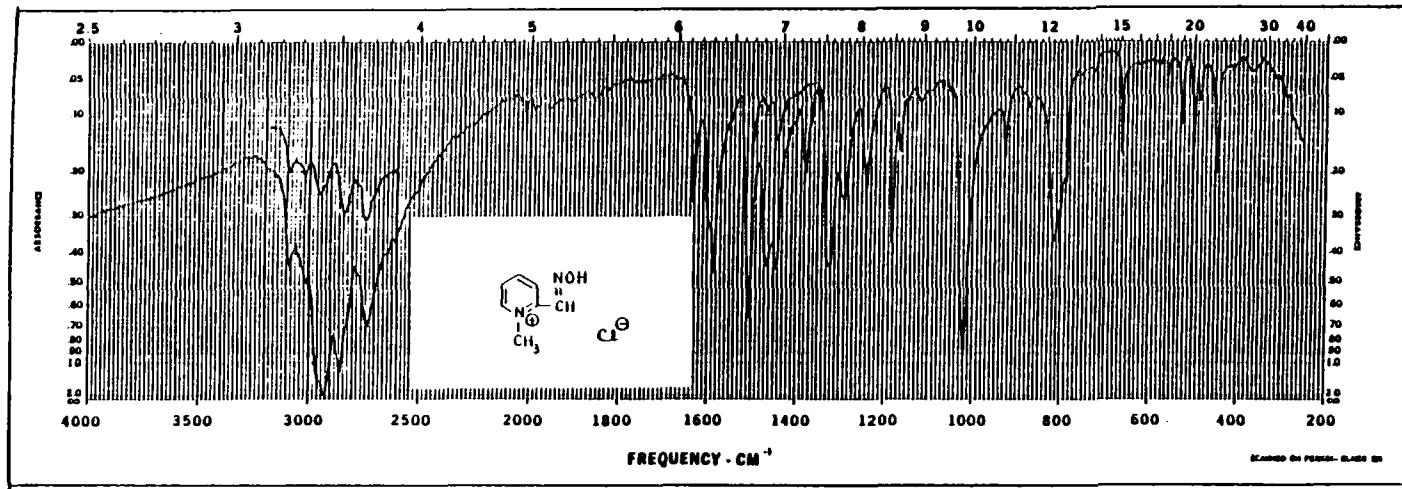


Fig. 6: Infrared Absorption Spectrum of Pralidoxime Chloride in Split Mull

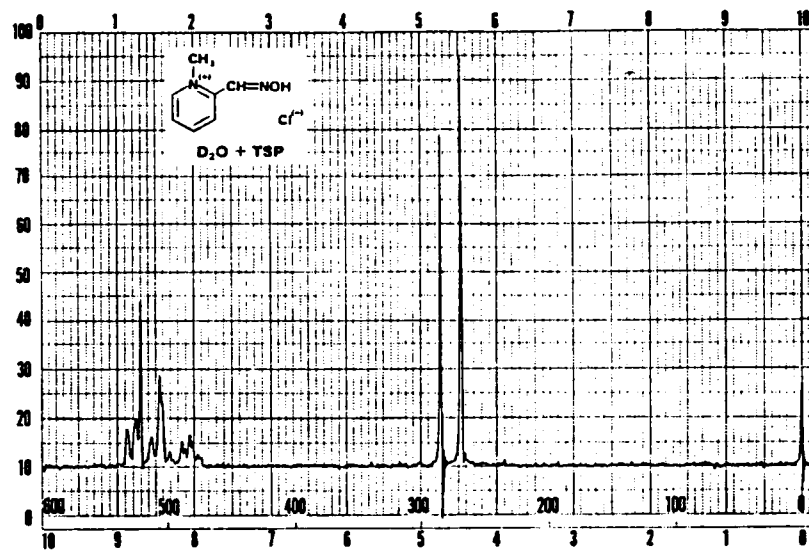


Fig. 7: NMR of Pralidoxime Chloride

Filter bandwidth: 4 Hz  
 Sweep time: 250 sec  
 Sweep width: 500 Hz  
 Sweep offset: 60/360 Hz  
 Spectrum amp: 20/50  
 Integral amp: 80 (spec. amp. 12.5)  
 Solvent: DMSO-d<sub>6</sub> at 80°C

# ASSIGNMENTS

a	<u>4.50</u>	h	<u>2.58 DMSO-d<sub>6</sub></u>
b	<u>8.13</u>	i	<u>          </u>
c	<u>8.41</u>	j	<u>          </u>
d	<u>8.61</u>	k	<u>          </u>
e	<u>8.71</u>	l	<u>          </u>
f	<u>9.13</u>	m	<u>          </u>
g	<u>12.00-13.50</u>	n	<u>          </u>

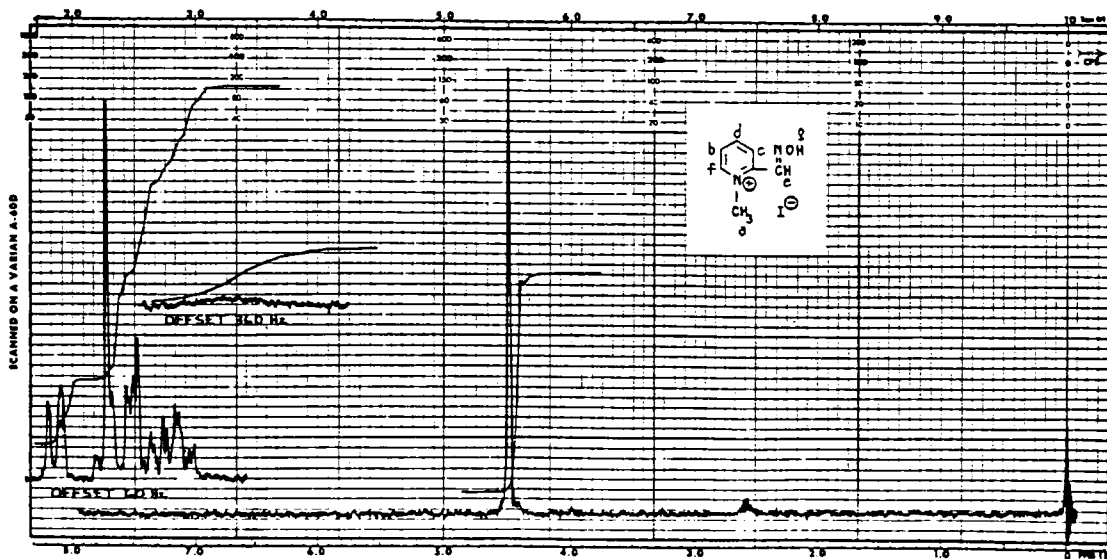


Fig. 8: NMR of Pralidoxime Iodide

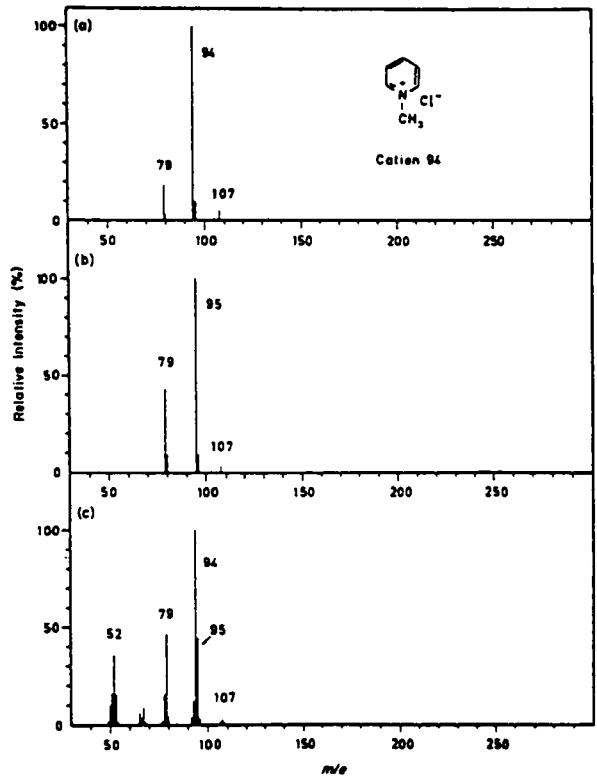


Fig. 9: Mass Spectra of 1-methyl pyridinium chloride.  
a) FD; b) FI; and c) EI

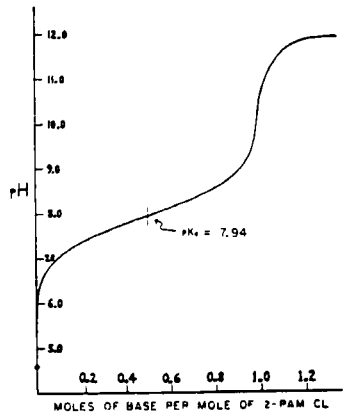


Fig. 10: Titration Curve of Neutralization  
of Pralidoxime Chloride

## 2.7. Synthesis: (Kondritzer et al., 1961).

### 2.7.1. Preparation:

The oxime salts are commonly prepared by adhering to one of the general procedures as follows: The silver salt and methyl pyridinium-2-aldoxime iodide (2-PAM iodide) are dissolved in minimum volume of water and mixed thoroughly. The total content of 2-PAM iodide is slightly in excess of that required for stoichiometry. The mixture is agitated for 30 minutes following which the silver iodide is filtered. The filtrate is evaporated to dryness with a steam bath under reduced pressure employing a rotating evaporator. The residual is recrystallized from 95% ethanol.

The chloride salt can also be prepared by means of ion exchange resonance employing column techniques using two strong anionic exchange resins. A molar solution of sodium chloride is passed through both columns and the resins subsequently washed with water until free of halides. Solutions of 2-PAM iodide are added and the columns washed with water as previously described. The fractions of the eluate containing halide are collected and the chloride salt is isolated and purified from 95% ethanol.

### 2.7.2. Commercial Preparations:

Several procedures for the commercial production of methylpyridinium-2-aldoxime chloride have been reported (US Patent, 1964a,b,c; Ellin, 1964). These methods primarily revolve around conversion of picolinal to its oxime which is subsequently quaternized with dimethyl sulfate. Metathesis of the resulting pralidoxime methosulfate with hydrochloric acid is pralidoxime chloride.

#### Procedure #1:

An aqueous solution of 10 mL of 1-methyl-2-picolinium chloride is covered with 50 mL of  $C_6H_6$  in a nitrogen atmosphere, cooled to  $5^{\circ}C$  and treated with a solution of 6 g of NaOH and 6 mL of water. The aqueous phase is separated and the  $C_6H_6$ -solution is slowly added with good agitation at approximately  $10-15^{\circ}C$  in a nitrogen atmosphere to a solution of methyl nitrite in 200 mL of benzene containing 1 mL of DMF and the mixture stirred for 1 h at ambient temperature. The reaction mixture is then treated with a solution of 5 mL of concentrated HCl in 10 mL of water, the aqueous phase separated and the organic phase extracted with two 10 mL portions of water. The aqueous phases are combined, evaporated and the residue collected in 60 mL of absolute alcohol, boiled with activated charcoal, filtered and washed with 10 mL of absolute alcohol,

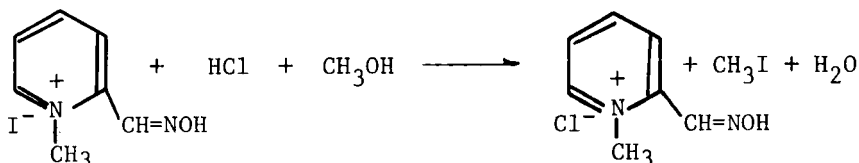
yielding a first crop of 1.5 g of pralidoxime chloride melting at 216-221°C with decomposition. The second crop of 1.4 g of pralidoxime chloride with a melting point of 217-220°C, with decomposition, is finally obtained.

Procedure #2:

Ten g of 2-pyridine aldoxime methyl-methylsulfate is dissolved in 8 mL of concentrated HCl and 80 mL of isopropanol is added. Crystals appear quickly, and are filtered off after 2 h at room temperature and washed with acetone. The product with a melting point of 229-230°C is yielded up to 84%. The mixture, when chilled in an ice bath before filtering off the product, yields almost 91% pralidoxime chloride having a melting point of 226-227°C.

Procedure #3:

Ellin (1964) described a procedure for the commercial preparation of pralidoxime chloride employing a recycling process by simply heating the corresponding iodide salt with methanolic hydrogen chloride. The iodide salt is added to methanolic HCl and the resulting mixture is heated for 45 minutes. The corresponding chloride salt (pralidoxime chloride) is isolated in yields of about 90%. A byproduct of this reaction is methyl iodide, which probably forms by the displacement of the iodide ion of the iodide salt (starting reagent) by chloride ion and the subsequent reaction of hydrogen iodide with methanol. The methyl iodide can be recycled to form the iodide salt, the starting product of the reaction. The pralidoxime chloride which is formed resultant to this process requires no further recrystallization and is approximately 100% pure.



### 3. ANALYTICAL

Pralidoxime chloride and its major degradation products can be identified and/or quantified by employing various analytical methods (refer Table III).

TABLE III

Various Analytical Methods employed for Identification and/or Quantification of Pralidoxime Chloride  
and its Major Degradation Products in Various Biological and Nonbiological Material

Method	Whole Blood	Plasma	CSF	Urine	Feces	Others	References
Colorimetry	X					K,L,S,B	Blom, 1976; Csaky, 1948
UV Spectrophotometry	X	X		X	X	BT  SM,L,K As AqS	Groff and Ellin, 1969 Creasey and Green, 1959; Zvirblis, 1959; Berglund, <u>et al.</u> , 1962 May, <u>et al.</u> , 1965 Lehman and Bloch, 1965 Ellin and Kondritzer, 1969
IR						KBr pellet, Nm,Sm	Pouchert, 1975: Sadtler Standard Spectra, 1967
NMR						D <sub>2</sub> O + TSP	Pouchert, 1983
Mass Spectrometry* (Field Desorption)							Elfinn, <u>et al.</u> , 1978
Polarography						VBM	Meites and Zuman, 1974

continued



TABLE III CONTINUED

Method		Whole Blood	Plasma	CSF	Urine	Feces	Others	References
HPLC							As	Fyhr, <u>et al.</u> , 1986
							Is	Prue, <u>et al.</u> , 1983
<hr/>								
Ion-Pair								
Reversed-Phase HPLC							DW	Benschop, <u>et al.</u> , 1981
Scintillation Spectrometry	<sup>14</sup> C	X		X	X	X		Calesnick, <u>et al.</u> , 1967
	<sup>3</sup> H	X		X	X	X		
	<sup>14</sup> C						RT,B	Harris, <u>et al.</u> , 1971
<hr/>								
Voltametry								Fyhr, <u>et al.</u> , 1986
<hr/>								
Fluorescence Spectrometry								Sunshine, 1981

\*For the pyridinium nucleus.

K (kidney), L (liver), S (spleen), B (brain), BT (biological tissue), SM (skeletal muscle), Nm (Nujol mull), Sm (split mull), VBM (various buffer media), AS (acidic solutions), IS (injectable solutions), DW (distilled water), RT (retinal tissue), AqS (aqueous solutions).

### 3.1. Colorimetric Method:

Pralidoxime chloride has been analyzed for the parent drug employing colorimetry (Blom, 1976; Csaky, 1948; Burger, et al., 1986).

### 3.2. Spectrophotometric Methods:

The ultraviolet absorption spectra for pralidoxime chloride in 0.1 N HCl and in 0.1 N NaOH are illustrated in Figs. 2 and 3, respectively (May, et al., 1965). Ultraviolet spectra for pralidoxime chloride at various pH values ranging from 6-10 are illustrated in Fig. 4 (May, et al., 1965). The infrared absorption spectra for pralidoxime chloride in a potassium bromide disk and in a Nujol mull are depicted in Fig. 6 and 7, respectively (May, et al., 1965). It is recommended that the infrared spectra of the disk should be obtained within 1 h after its preparation.

### 3.3. Chromatographic Methods:

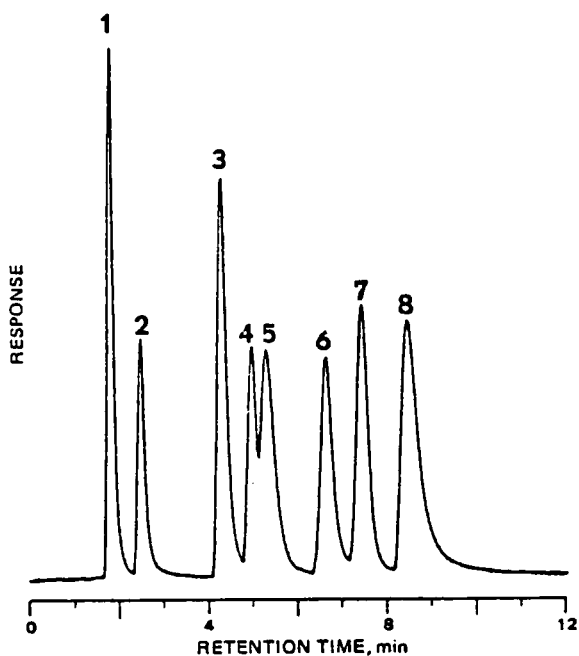
Several High-Performance Liquid Chromatographic (HPLC) methods have been employed for determination of pralidoxime chloride and its major decomposition products in aqueous solutions (Prue, et al., 1983; Fyhr et al., 1986).

#### System #1:

The HPLC system consists of a mobile-phase pump operated at 0.8 mL/min (1600 psi), sample injector set for a 15  $\mu$ L injection volume, a variable-wavelength UV detector at 270 nm, a precolumn positioned between the pump and the injector, a precolumn positioned between the pump and the injector, and an analytical reversed-phase C-18 column. Areas under the chromatographic peaks are measured by electronic integration. The mobile phase consists of 52% acetonitrile and 48% of an 0.001 M tetraethylammonium chloride. The chromatographic data are acquired at room temperature. This system can be employed for the identification of pralidoxime chloride (I) and its major decomposition products: 2-carboxy-N-methylpyridinium chloride (II), N-methyl-2-pyridone (III), 2-carbamoyl-N-methylpyridinium chloride (VIII), 2-hydroxymethyl-N-methylpyridinium chloride (X), and 2-cyano-N-methylpyridinium chloride (XI). Recovery of pralidoxime chloride from a spiked placebo sample averages 99.9%. A typical chromatogram of a synthetic mixture of I-XII is shown in Fig. 11.

#### System #2:

The HPLC system consists of a reversed-phase column, a mobile phase of 2 mM sodium octyl sulfonate, 50 mM



**Fig. 11:** Chromatogram of a synthetic mixture of I-XII. Key: (1) II, (2) III and IV, (3) V, (4) VI and VII, (5) VIII, (6) IX and X, (7) I and (8) XI. Compound XII is not shown (retention time = 27.4 min).  
IV - 2-cyano-1-methyl-4-pyridone,  
V - pyridine-2-aldoxime,  
VI - syn-pralidoxime chloride,  
VII - 2-formyl-N-methylpyridinium chloride,  
IX - 2-aminomethyl-N-methylpyridinium chloride and  
XII - N-methyl-pyridinium chloride. Refer text for remaining decomposition products.

$\text{Me}_4\text{NCl}$ , 20 mM acetic acid and 10% methanol in distilled water; a pump and a flow/rate of 0.8 mL/min. Samples are injected employing a Rheodyne 7120 injection valve with a 20  $\mu\text{L}$  loop. Detection is performed with a variable wavelength UV detector. The chromatograms are recorded with a computing integrator and pen recorder. Peak heights are evaluated at the wavelength for their absorbance maximum (between 200-300 nm).

### 3.4 Polarographic Methods:

Pralidoxime chloride has been reported to be identified by polarographic methods in various buffer media (Meites and Zuman, 1974). The characteristic potential values employing various electrodes for pralidoxime chloride is presented in Table-IV.

TABLE IV

Polarographic Data for Pralidoxime Chloride,  
Characteristic Potential Values:

Electrodes	$E_p$	$E_{1/2}$	$E_{1/2}$	$E_s$
SLE:	0.70F	-	(-2.14)-0.406	(-1.55)-(-1.18)
CdSE	-	0.87	-	-
NCE	0.75	-	-	-
Ag/AgCl 0	-	-	-2.751	-

SLE: saturated calomel; CdSE: Cadmium sulfate;

NCE: normal calomel.

### 3.5. Other Methods:

Pralidoxime chloride has been reported to be identified by voltametry, scintillation spectrometry, radiochromatography, and fluorescent spectrometry (Harris, et al., 1971; Sunshine, 1981; Fyhr, et al., 1986; Galesnick, et al., 1987;).

## 4. STABILITY CONSIDERATIONS:

### 4.1. Degradation Pathway(s):

The degradation of pralidoxime chloride has been studied extensively in acidic media. Two routes of degradation of pralidoxime salts (chloride, iodide and methanesulfonate) have been reported. The primary consideration of the routes of degradation revolve around pH of the medium containing any given pralidoxime salt.

At pH values below 4, a hydrogen-ion catalyzed hydrolysis to yield the appropriate aldehyde and hydroxylamine has been reported (Ellin and Esterday, 1961; Ellin *et al.*, 1962; Berkman, *et al.*, 1963; Ellin and Wills, 1964; Cristensson, 1974). Subsequently, the aldehyde is oxidized to the corresponding acid (Cristensson, 1974).

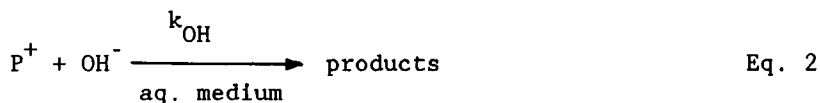
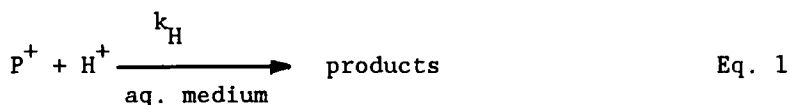
A hydroxyl-ion catalyzed dehydration reaction occurs at pH values above 4. This degradation pathway results in the formation of cyanide which subsequently hydrolyzes to either N-methyl-pyridone and/or cyanide ion, N-methyl-2-2-carbamidopyridine (Ellin, 1958; Creasey and Green, 1959; Ellin *et al.*, 1962; Barkman, *et al.*, 1963; Ellin and Wills, 1964; Fan, *et al.*, 1964; Carter, *et al.*, 1968; Sidell, 1976).

The concentration of the pralidoxime salt in question has been low in most of the reported literature. Additionally, the degradation products have been identified predominantly in concentrated acidic solutions (Prue, *et al.*, 1983; Fyhr, *et al.*, 1986; Fyhr and Brodin, 1987). The observed degradation pathways for pralidoxime chloride are illustrated in Fig. 12.

#### 4.2. Reaction rate and Reaction equations:

The degradation of pralidoxime chloride occurs essentially via hydrogen ion and hydroxyl ion catalysis. The mechanism of decomposition reactions are illustrated in Fig.12.

Pralidoxime, as the free oxime, exists as a cation ( $P^+$ ) and the basic or oximate species exists as a zwitter ion ( $P^+$ ) (Connors, *et al.*, 1986). Pralidoxime undergoes the following reactions depending on the decomposition resultant to either hydrogen ion or hydroxyl ion catalysis;



where,  $k_H$  and  $k_{OH}$  represent the specific reaction rate constants respectively. The specific acid catalysis of the positively charged pralidoxime ion is represented in Eq. 1, while, Eq. 2 represents the specific base catalysis. Consequently, the corresponding decomposition reaction rate equation is:

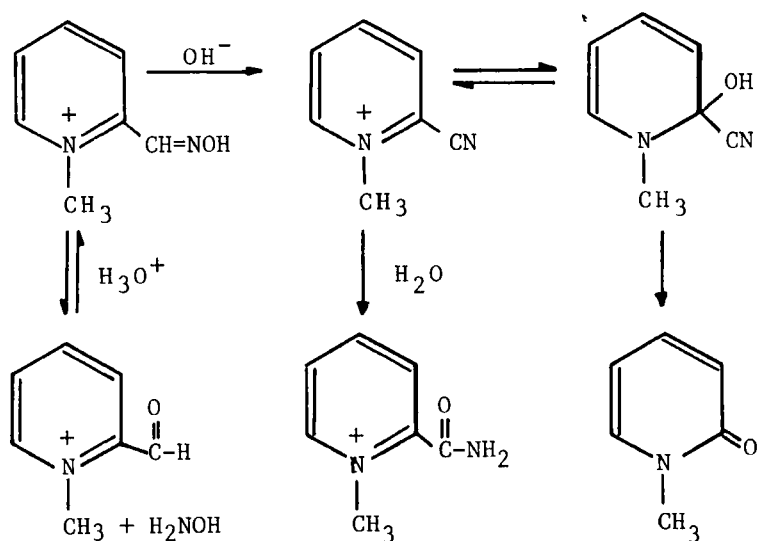


Fig. 12: Decomposition pathways Observed for Pralidoxime Chloride in Aqueous Media

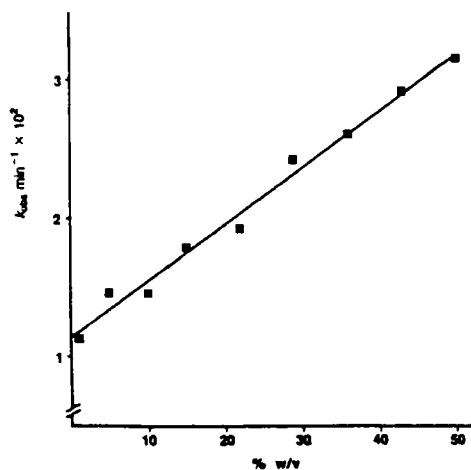


Fig. 13: Observed Decomposition Rate Constants at Different Concentrations of Pralidoxime Chloride at an initial pH of 4.0 at  $120^\circ\text{C}$

$$\text{Reaction rate} = k_H[H^+]\cdot[P^+] + k_{OH}[OH^-]\cdot[P^+] \quad \text{Eq. 3}$$

The ionization of the oxime group,  $P^+$ , considering the  $pK_a$  to be approximately 7.8, Eq. 3 can be written in terms of total pralidoxime concentrations. Thus, it permits derivation of the pseudo-first-order-rate constant for decomposition as given in Eq. 4.

$$K_{obs} = [k_H \cdot [H^+]^2 / (k_a + [H^+])] + [k_{OH} \cdot K_w / (k_a + [H^+])] \quad \text{Eq. 4}$$

where,  $k_H = 5.8 \times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$  and  $k_{OH} = 251 \text{ M}^{-1} \text{ min}^{-1}$  at  $37^\circ\text{C}$ . A similar expression which is kinetically equivalent to Eq. 4 can be obtained following the attack of a water molecule on the zwitter ion,  $P^\pm$ , instead of the attack of hydroxyl ion on the cation,  $P^+$  (Ellin, *et al.*, 1962).

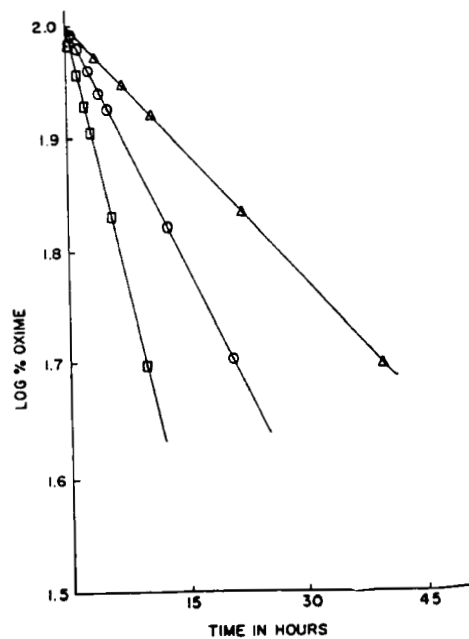
The degradation rate constant for pralidoxime chloride is dependent on the concentration employed in the study. An increased decomposition rate is observed with increasing concentration as illustrated in Fig. 13. However, the pH decreases more rapidly in the more concentrated solutions than in the dilute ones (Fyhr, *et al.*, 1986).

#### 4.3. Decomposition Rate - pH profile:

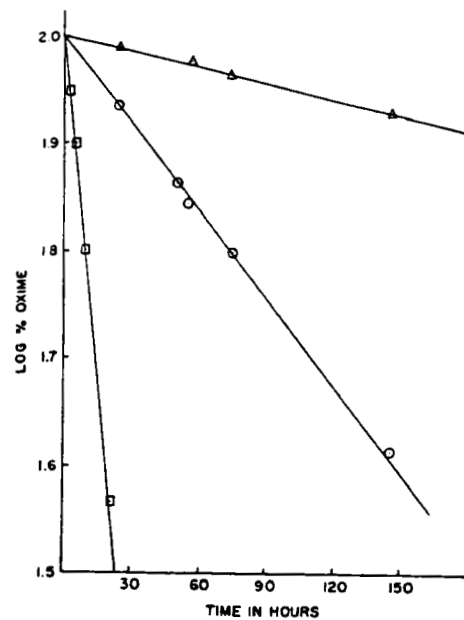
The observed rate of deterioration of pralidoxime salt (iodide or chloride) at constant pH and temperature adheres to a typical pseudo-first-order process with respect to drug concentration. Figure 14 illustrates typical pseudo-first-order rate plots for decomposition of pralidoxime salt under various conditions of experimentation.

The hydrolysis of pralidoxime is due to hydrogen ion catalysis of the protonated species,  $P^+$ , or an attack of water molecule on the zwitter ion. A pH independent region exists between pH values 8-13 which can be primarily attributed to the ionization of the oxime group which initiates the base catalyzed hydrolysis of pralidoxime, i.e., the zwitter ion is not subjected to hydrolysis by the hydroxyl ion. The pH rate profile for pralidoxime is depicted in Fig. 15.

It has also been reported that the decomposition rate constants of pralidoxime chloride exhibit an ascending slope from lower to higher pH values. Consequently, since the degradation rate decreases with pH, the slope of the concentration profile is small. Furthermore, it seems probable that the rate constant resultant to hydroxyl ion attack far exceeds the rate constant resultant to hydrogen ion attack and that a water or uncatalyzed mechanism influences the reaction at low pH in concentrated solution (refer Fig. 16a,b).



**A**



**B**



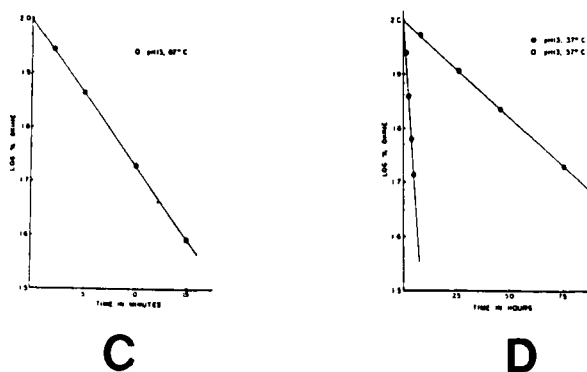


Fig. 14: Typical First-Order Rate Plots for Decomposition of Pralidoxime. A: Acid catalyzed decomposition at 57°C  $\Delta$ , 0.096 N;  $\circ$ , 0.191 N;  $\square$ , 0.383 N perchloric acid, B: Decomposition at  $\Delta$ , pH 5,  $\circ$ , pH 6, and  $\square$ , pH 7 at 57°C, C & D: Decomposition at pH 13 at various temperatures

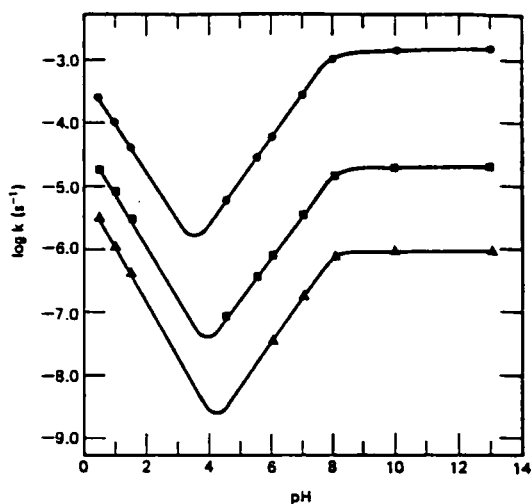


Fig. 15: pH-Rate Profile for Degradation of Pralidoxime at Differing Temperatures ( $\bullet$ , 37°C;  $\blacksquare$ , 57°C; and  $\blacktriangle$ , 87°C)

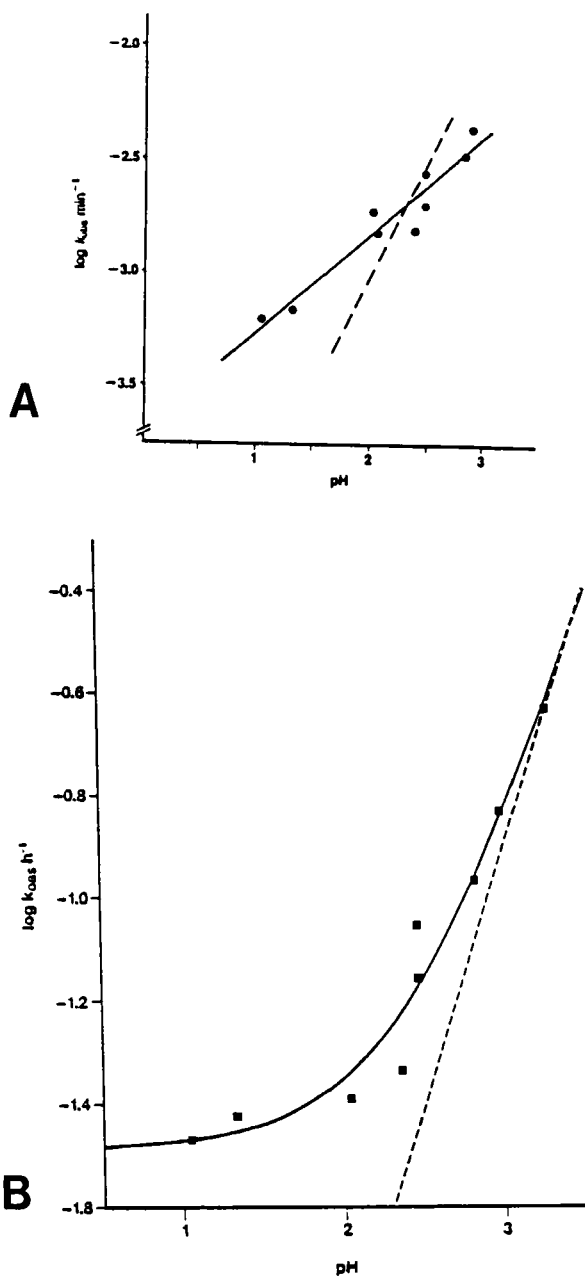


Fig. 16: pH Profile for Pralidoxime Chloride, 430 mg/mL, at 120°C. A: from Fyhr, *et al.*, 1986, B: from Fyhr and Brodin, 1987

#### 4.4. Ionic Strength:

The rate constant for decomposition of pralidoxime chloride exhibits a direct dependence on ionic strength. Fyhr and Brodin (1987) investigated the influence of concentration and ionic strength on the decomposition rate of pralidoxime chloride employing concentrations between 10 and 500 mg/mL at 120°C. The decomposition obeyed modified Bronsted-Bjerrum equation (Florence and Attwood, 1981):

$$\log k_{\text{obs}} = \log k_I - 1.02 \cdot Z_A \cdot Z_B \cdot \frac{\sqrt{I}}{1 + \beta \cdot \sqrt{I}} \quad \text{Eq. 5}$$

where,  $k_I$  is the rate constant at finite ionic strength,  $Z_A$  and  $Z_B$  the valence of the reacting species and  $\beta$ , a constant depending on the ionic diameter of the reacting species. Since, the mechanism of decomposition appears to change between 5 and 15% pralidoxime chloride, the hydrogen ion catalyzed mechanism is predominantly active below 5%. The decomposition of pralidoxime chloride exhibiting its dependence on ionic strength is illustrated in Fig. 17 (Fyhr and Brodin, 1987).

#### 4.5. Temperature Effects and Activation Energy:

The decomposition of pralidoxime chloride exhibits temperature dependence (Ellin, et al., 1962; Lehman and Bloch, 1965; Ellin, 1982; Fyhr and Brodin, 1987). Good linearity is observed when the decomposition data is analyzed for Arrhenius relationship. Figures 18-19 depict the temperature dependence of the rate constant for decomposition of pralidoxime salts (chloride, mesylate) at pH values ranging from 1 - 10.9 for mesylate salt and at pH values ranging from 3-4 for chloride salt.

The activation energy, calculated subsequent to Arrhenius study, associated with the specific acid and specific base catalysis of pralidoxime hydrolysis is presented in Table V (Ellin et al., 1962; Fyhr and Brodin, 1982). No significant differences in activation energies are observed between acid catalyzed and base catalyzed degradation processes.

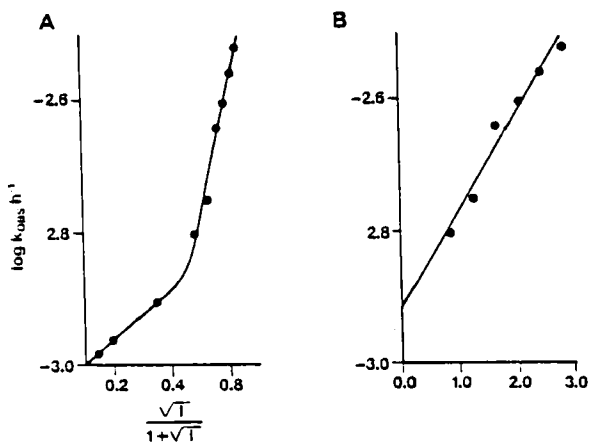


Fig. 17: Dependence on Ionic Strength. A: Modified Bronsted Bjerrum equation. B: Straight relation 15-50%

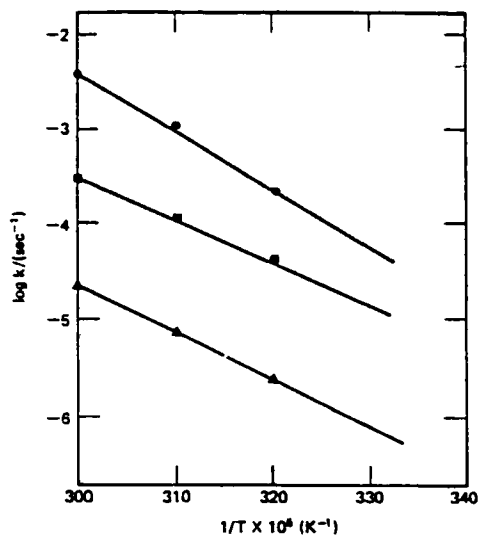


Fig. 18: Arrhenius Plot for Pralidoxime salt (mesylate) at different pH values (●, 10.9; ■, 1.0; and ▲, 5.0)

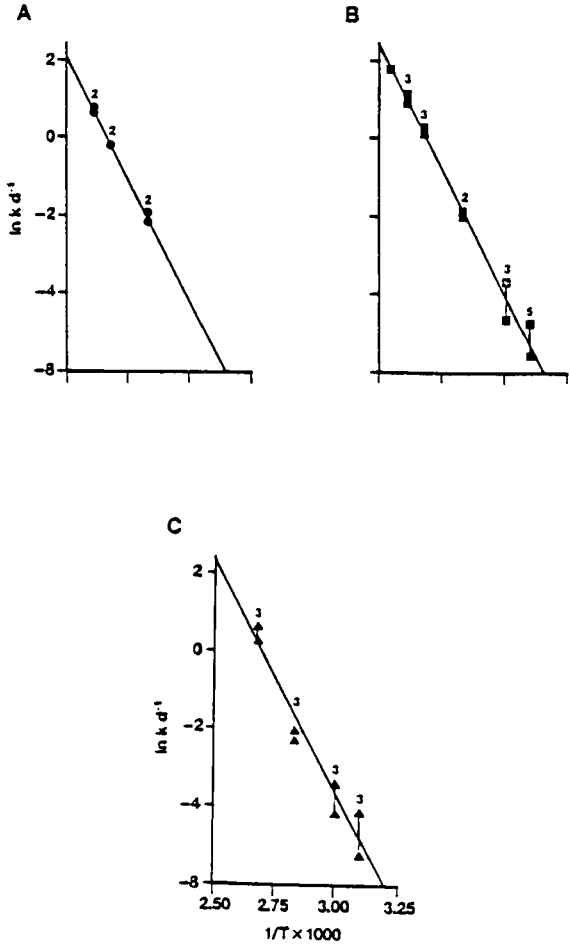


Fig. 19: Temperature Dependence, Pralidoxime Chloride 480 mg/mL, A: pH 3, B: pH 3.5, C: pH 4

TABLE V

Activation Energies for the Rate Constants Describing the Degradation of Pralidoxime

<u>Rate Constant</u>	<u>Ea(KCal/mol)</u>
$K_H$	17.4
$K_{OH}$	17.0

#### 4.6. Stability of Pralidoxime Chloride Formulations:

The possible degradation products of pralidoxime chloride are given in Fig. 12. The hydroxyl ion attack results in the production of the appropriate cyanide (Fan, *et al.*, 1964). The amount of cyanide formed in solutions of different pH values maintained at different temperatures over prolonged periods is presented in Table VI. No significant relationship is observed between the amount of cyanide formed and the amount of pralidoxime degraded.

TABLE VI

Cyanide Concentration ( $\mu\text{g/mL CN}^-$ ) Formed in Aqueous Solutions at Different pH and Temperature Values from 23  $\mu\text{g/mL}$  Solutions of Pralidoxime.

pH	Temperature ( $^{\circ}\text{C}$ ), h					
	40	h	50	h	60	h
4	0	210	0	209	0	80
5	0	229	0.013	120	0.035	60
5.95	0.01	70	0.043	70	0.35	60
6.95	0.29	70	0.65	50	1.31	50
9.10	0.83	40	1.44	50		

Lehman and Bloch (1965) studied the effect of aging of 5% aqueous pralidoxime solutions (chloride and iodide salts) at an initial pH of 5. The chloride salt was more stable than the iodide salt. The percentage of each salt remaining after 3 years is illustrated in Table VII. The authors suggest that the more is rapid deterioration of the iodide salt occurs due to the stagnation of pH at 6 which is above the optimal pH of 4.4. However, the pH of the chloride

salt drops progressively to pH values where pralidoxime has greater stability. The differing pH shifts remain unexplained.

TABLE VII

Percentage of 2-PAM Salts Remaining at Various Times.

Age of Solution (months)	Percent of Value at Zero Time	
	2-PAM Chloride	2-PAM Iodide
0	100	100
12	97.1	92.5
24	94.3	86.3
36	93.1	81.5

Ellin (1982) studied the effect of glass, metal, plastic and rubber stoppers on the stability of concentrated and dilute solutions of pralidoxime chloride. The rate of degradation of concentrates of pralidoxime at 60, 70 and 81°C conforms to a first order pathway. Linear relationship was observed when the decomposition rate data was analyzed for Arrhenius plot with resultant energy of activation of 26.7 KCal/M. The buffering of solutions of pralidoxime chloride exhibits no influence on stability, as well (Barkman, 1963). The shelf-life of aqueous solutions of pralidoxime chloride in various containers (glass, plastic and metal) was determined (Ellin, 1982). The calculated shelf-life for 50% aqueous solutions of pralidoxime chloride for 10% and 25% degradation at temperatures ranging from 10-50°C is presented in Table VIII.

TABLE VIII

Calculated Shelf-Lives (years) for pralidoxime chloride in Glass Ampuls (unbuffered, pH = 3.65).

Temperature (°C)	10% Degrad.	25% Degrad.
10	37.0	101.0
15	16.0	44.0
20	7.3	20.0
25	3.4	9.3
30	1.6	4.4
35	0.78	2.1
40	0.39	1.1
45	0.19	0.54
50	0.10	0.28

The degradation rates of 50% aqueous solution of pralidoxime chloride were similar in plastic and glass cylinders sealed with neoprene stoppers and more rapid in metal cylinders at 40°C. The degradation at 45 and 60°C in containers with 30% solutions of pralidoxime chloride stoppered with either neoprene or butyl rubber exhibited decrease in pH more rapidly for containers sealed with the latter than the former (Calesnick, et al., 1967).

Evidently, it appears that pH control is the critical factor in the stabilization of aqueous solutions of pralidoxime. Provided that the pH and storage temperatures are stringently controlled, adequate shelf-lives of pralidoxime solutions are obtained with relative ease.

## 5. BIOPHARMACEUTICS AND PHARMACOKINETICS

The therapeutic concentration for pralidoxime chloride has been reported to be 4 ug/mL (Sidell, et al., 1969, 1971; Holland, et al., 1972; Vajvodic and Maksimovic, 1972). However, the original investigation by Sundwall (1961) indicated a concentration of 4 ug/mL to be at the minimal effective level. The range of effective therapeutic concentrations is 4 ug/mL to 12 ug/mL.

### 5.1. Absorption:

Pralidoxime chloride is administered orally and intramuscularly. Absorption of pralidoxime chloride is variable and incomplete following oral administration (Sidell, et al., 1969). Peak plasma oxime concentrations are reached after 2-3 h after oral administration, 5-15 min after IV administration and 10-20 min. after IM administration of pralidoxime chloride. Considerable interindividual variation in plasma oxime concentration is reported in adults receiving the same dose. Multiple dosing diminishes the variability but is still significant. Intravenous pralidoxime chloride doses of 7/5-10 mg/Kg are required to produce plasma oxime concentrations of 4 g/mL or greater at 1 h after administration. Similar IM doses are required to achieve initial plasma concentrations of 4 ug/mL or greater, and only IM doses of 10 mg/kg are needed to maintain plasma oxime concentrations at 4 ug/mL is greater for 1 h.

### 5.2. Distribution:

Clinical responses observed by Lotti and Becker (1982) suggest that therapeutic amounts distribute to the brain, however, this observation is not confirmed by measurement. Pralidoxime is distributed throughout the extracellular water, due to its quaternary ammonium structure. Pralidoxime does not readily penetrate the cornea



following systemic or topical administration, however, therapeutic concentrations are reportedly achieved in the eye following subconjunctival injection. (AHFS, 1987). Sidell, et al., (1972a,b) have reported that the volume of distribution for pralidoxime is greater than body volume and that at steady state, has an apparent volume of distribution of 0.775 to 0.815 L/Kg. Pralidoxime is not appreciably bound to plasma proteins (AHFS, 1987).

### 5.3. Elimination:

The exact metabolic fate of pralidoxime has not been completely elucidated, however, the drug is believed to be metabolized in the liver. Pralidoxime is reported to be excreted unchanged in the urine in the range of 20-99% (Sidell, et al., 1969; Sidell and Groff, 1971; Sidell, et al., 1972 a,b; Vajvodic and Maksimovic, 1972). Pralidoxime is reported to have a half-life of 1.23 h with a range of 1.2-2.7 h. Furthermore, pralidoxime is reported to have a renal clearance ratio of 4:1 indicating active secretion by renal tubular cells (Sidell, et al., 1972a) with a renal clearance of about 600-700 mL/min. Swartz and Sidell (1974) suggest that pralidoxime is secreted as an organic base, however, they further note that both alkalinization and acidification of urine reduces the excretion of the compound.

## 6. METHODS OF ANALYSIS IN BIOLOGICAL FLUIDS:

Several analytical methods have been reported in the literature, identification, and quantitation of pyridinium aldoxime compounds in various biological materials such as blood, plasma, urine, feces, CSF and biological tissues (refer Table III). Of the different procedures reported in the literature, two procedures are routinely and frequently adopted.

### Procedure #1 (Groff and Ellin, 1969):

A rapid and accurate method for analyzing pyridinium oximes in plasma, urine and whole blood is described which is completely automated and employs spectrophotometric method. The analytic system consists of the following modules:

1. automated sampler,
2. proportioning pump,
3. dialyzer with two sets of dialyzing plates
4. a recording Beckman DB spectrophotometer, and
5. a recorder.

The wavelength of maximum absorption in 0.01 N NaOH is obtained at 335 nm for pralidoxime chloride. Figure 20

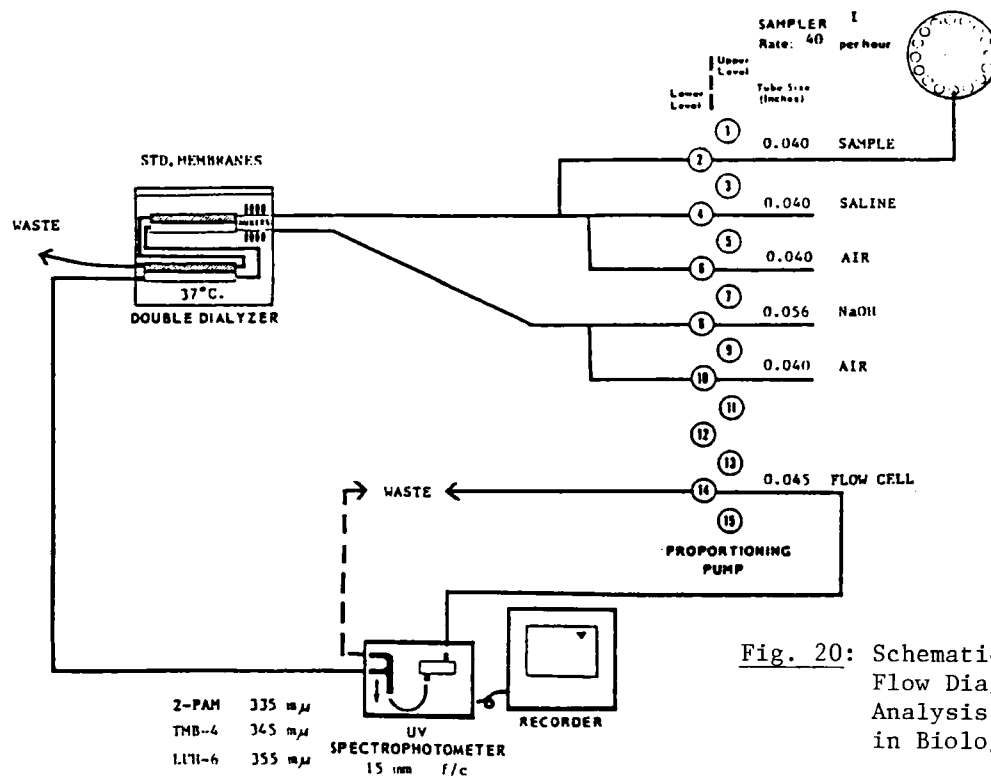


Fig. 20: Schematic Illustration of the Flow Diagram for Automated Analysis of Pyridinium Oximes in Biologic Fluids

illustrates a flow diagram of the automated method including all pump-tubing sizes. The sample is drawn from a liquid sampler and is diluted with an equal volume of saline containing 0.5% v/v Brij 35. The sample stream is segmented with air and enters another mixing coil in the dialyzer. The sample stream and the recipient stream, which equilibrate at 37°C, now pass through the continuous helical channels of the dialyzing plates, which are mated and separated by a membrane. Concentration of the substance to be measured that is proportional to the concentration in the original sample passes into a recipient stream before the streams leave the dialyzer. The sample stream now goes to waste, while the recipient stream, after being debubbled, enters the flow cell of the spectrophotometer. The stream is drawn through the flow cell by the pull-through pump tube at a controlled rate while the excess solution and air flow go to waste. Absorbance values are continuously recorded on a strip chart which are subsequently used either to calibrate or to quantify the concentration of the drug in the sample.

Procedure #2 (Benschop, et al., 1981):

An ion-pair reversed-phase HPLC method for the determination and quantification of pyridinium aldoxime compounds in biological material (blood) is presented. The system involves a 5- $\mu$ m C<sub>18</sub> silica gel stationary phase. The eluent consists of methanol, acetic acid buffer (pH 4.80), a counter ion (perchlorate or n-octane sulfonate) and a surfactant. The compounds are detected spectrophotometrically at 304 nm. Detection limits in blood are satisfactory (0.5-1  $\mu$ M).

## 7. SUMMARY

Pralidoxime chloride is predominantly prepared by conversion of picolinal to its oxime which is subsequently quaternized with dimethyl sulfate following which is treated with HCl resulting in the production of pralidoxime chloride. Pralidoxime is identified and quantified by several methods, however, the spectrophotometric method is more commonly employed.

The physical-chemical characteristics of pralidoxime chloride are outlined in this profile. This drug substance, due to its chemical structural configuration, undergoes decomposition resultant to both hydrogen-ion as well as hydroxyl-ion attack. Consequently, the environmental pH and the ambient temperature are important considerations in the evaluation of the stability of pralidoxime chloride.

Biopharmaceutic and pharmacokinetic considerations suggest that the absorption of pralidoxime is variable and incomplete following oral administration. The therapeutically effective concentrations range between 4-12 ug/mL. The drug is rapidly and almost completely excreted in urine as unchanged drug. The biological half-life of pralidoxime in humans with normal kidney function ranges between 0.8-2.7 h.

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NOTES:

1. The notation 'u' in this profile denotes 'micro-', e.g., ug-micrograms, etc.
2. Figures 2-10, 12-20 and Tables V-VIII are reproduced by permission of copyright owners.

## **SULFADOXINE**

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### **1. Description**

**1.1 Name, Formula and Molecular Weight**

**1.2 Appearance, Color, Odor**

### **2. Physical Properties**

**2.1 Infrared Spectrum**

**2.2 Ultraviolet Spectrum**

**2.3 Optical Rotation**

**2.4 Melting Range**

**2.5 Solubility**

**2.6 Acidity**

**2.7 Crystal Properties**

### **3. Synthesis**

### **4. Stability and Degradation**

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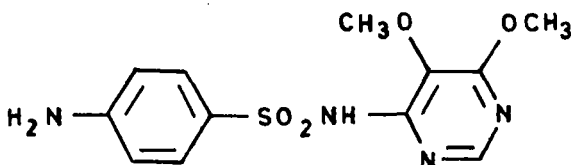
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## **9. References**

## 1. Description

### 1.1 Name, Formula and Molecular Weight

Sulfadoxine is a long-acting sulfonamide developed by scientists of F. Hoffmann-La Roche & Co. It is given with pyrimethamine in the treatment and prophylaxis of falciparum malaria resistant to other therapies. Other names given as synonyms for sulfadoxine include sulphadoxine, sulphormethoxine, sulformethoxine, sulforthomidine, sulphorthodimethoxine, sulfadimoxinum and Ro 4-4393.<sup>1</sup> Chemically sulfadoxine is N'-(5,6-dimethoxy-4-pyrimidinyl)sulfanilamide. The CAS Registry number is 2447-57-6. The most common proprietary name is Fansil.



C<sub>12</sub>H<sub>14</sub>N<sub>4</sub>O<sub>4</sub>S      Molecular Weight: 310.33

### 1.2 Appearance, Color, Odor

A white or creamy-white, crystalline powder; odorless.

## 2. Physical Properties

### 2.1 Infrared Spectrum

The infrared spectrum (KBr disc) of sulfadoxine exhibits principal peaks at wave-numbers 1583, 1161, 1596, 1315, 1091 and 1305.<sup>2</sup>

### 2.2 Ultraviolet Spectrum

The light absorption, in the range 230

to 350 nm, of a 2-cm layer of a 0.001 per cent w/v solution in 0.1N methanolic hydrochloric acid exhibits a maximum only at 267 nm; absorbance at 267 nm, about 1.1.<sup>3</sup>

### 2.3 Optical Rotation

Sulfadoxine exhibits no optical activity.

### 2.4 Melting Range

The melting range<sup>4</sup> of sulfadoxine is between 197° and 200°.

### 2.5 Solubility

Sulfadoxine is very slightly soluble (1 in 1000 to 1 in 10000) in water; slightly soluble (1 in 100 to 1 in 1000) in alcohol and in methanol; practically insoluble (1 in more than 10000) in ether; soluble in dilute mineral acids and in solutions of alkali hydroxides and carbonates.<sup>1,5</sup> Glycerol lower alkyl ethers (mono- and diethers) have been suggested as solvents for sulfonamides in pharmaceuticals.<sup>6</sup> A computer program for exact calculation of the basal solubility  $S_0$  and of the acid  $pK_a$  of sulfonamides according to the theory of least squares has been given in the digital computer language ALGOL60.<sup>7</sup>

### 2.6 Acidity

Sulfadoxine is acidic in nature. When 1 g of sulfadoxine is heated with 50 ml of carbon dioxide-free water at about 70° for five minutes and filtered after quick cooling to 20°, 25 ml of the filtrate requires for titration to pH 7.0 not more than 0.25 ml of 0.1N sodium hydroxide.<sup>3</sup>

### 2.7 Crystal Properties

Crystal and molecular structures of sulfadoxine have been determined using X-ray crystallographic methods by Shefter et al.<sup>8</sup> The crystal data are as follows:  $a = 8.873(2)\text{\AA}$ ,  $b = 8.784(1)\text{\AA}$ ,  $c = 18.938(5)\text{\AA}$ ,  $\alpha = 90.00^\circ$ ,  $\beta = 107.64(2)^\circ$ ,  $\gamma = 90.00^\circ$ ,  $d_o = 1.47\text{ g/cm}^3$ ,  $d_c = 1.47\text{ g/cm}^3$ ,  $Z = 4$ , and space group  $P2_1/c$ . Intensity data

were collected by the stationary counter-stationary crystal method on quarter-circle diffractometer, using nickel-filtered CuK radiation, out to a maximum  $2\theta$  value of  $11^\circ$ . The structure was solved by use of Patterson and Fourier syntheses and refined by least-squares to  $R = 0.071$  for 1774 reflections. The final atomic positional and thermal parameters of sulfadoxine are given in Table 1 and 2. The intramolecular bond distances and angles are shown in Figure 1. Sulfadoxine is capable of a variety of conformational states by means of rotation about the three bonds C1-S, S-N1 and N1-C7. The torsion angles about these

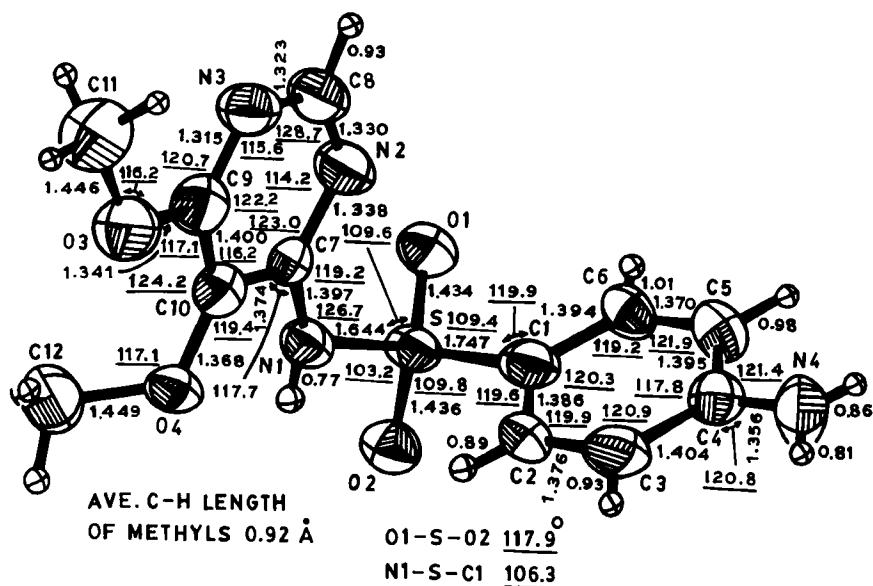


Figure 1 — Intramolecular bonding parameters for sulfadoxine. The molecules are seen with the bonds emanating from the sulfur atom oriented in the same perspective. The thermal ellipsoids for the nonhydrogen atoms are drawn at the 50% probability level.

Table 1— Positional and Thermal Parameters for Nonhydrogen Atoms together with Their Estimated Standard Deviations (in Parentheses)  $\times 10^4$ <sup>a</sup> of Sulfadoxine

Atom	x	y	z	b <sub>11</sub>	b <sub>22</sub>	b <sub>33</sub>	b <sub>12</sub>	b <sub>13</sub>	b <sub>23</sub>
S	916(1)	1456(1)	1014(1)	120(2)	97(2)	16(0.3)	-34(3)	19(1)	-11(1)
O(1)	726(4)	2490(4)	1566(2)	143(5)	125(5)	20(1)	-16(9)	31(4)	-29(4)
O(2)	-219(4)	252(4)	778(2)	135(5)	116(5)	22(1)	-82(9)	28(4)	-10(4)
O(3)	2674(4)	5562(4)	-1296(2)	198(7)	124(5)	27(1)	-48(10)	59(5)	3(4)
O(4)	1016(4)	2983(4)	-1114(2)	185(6)	93(5)	17(1)	-18(9)	15(4)	-15(4)
N(1)	757(5)	2389(4)	243(2)	141(6)	101(6)	15(1)	-39(10)	9(4)	-12(4)
N(2)	2415(5)	4468(5)	749(2)	178(7)	110(6)	20(1)	-72(11)	16(5)	-10(5)
N(3)	3292(5)	6118(5)	-50(2)	194(8)	107(6)	25(2)	-66(12)	32(5)	-5(5)
N(4)	7317(5)	-1179(6)	1980(2)	168(8)	182(8)	26(2)	128(13)	19(6)	13(6)
C(1)	2825(5)	697(5)	1301(2)	128(7)	91(7)	15(1)	-38(11)	23(5)	-14(5)
C(2)	3439(6)	27(6)	786(2)	138(8)	109(7)	15(2)	-3(12)	5(5)	-16(5)
C(3)	4926(6)	-609(6)	1012(3)	163(9)	109(7)	19(2)	10(13)	35(6)	-23(6)
C(4)	5829(6)	-605(5)	1761(3)	157(8)	87(7)	19(2)	3(12)	27(6)	9(5)
C(5)	5146(6)	13(7)	2270(3)	171(9)	146(8)	16(2)	17(4)	6(6)	-1(6)
C(6)	3677(6)	671(6)	2052(3)	155(9)	128(8)	16(2)	31(13)	27(6)	-3(6)
C(7)	1663(5)	3634(5)	156(2)	113(7)	68(6)	19(1)	1(11)	8(5)	4(5)
C(8)	3178(7)	5666(6)	598(3)	216(11)	117(8)	24(2)	-99(5)	10(7)	-23(6)
C(9)	2579(6)	5249(6)	-618(3)	133(8)	107(7)	23(2)	29(12)	29(6)	13(6)
C(10)	1711(5)	3960(5)	-546(3)	126(7)	77(6)	20(2)	15(11)	22(5)	3(5)
C(11)	3346(7)	7021(7)	-1386(3)	209(11)	148(9)	36(2)	-71(16)	76(8)	37(8)
C(12)	-231(7)	3592(8)	-1733(3)	178(10)	202(11)	28(2)	82(18)	-9(7)	-27(8)

<sup>a</sup>Temperature coefficient =  $\exp - (b_{11}h^2 + b_{22}k^2 + b_{33}l^2 + b_{12}hk + b_{13}hl + b_{23}kl)$ .

Table 2 — Positional and Thermal Parameters for Hydrogens of Sulfadoxine

Atom	$x \cdot 10^3$	$y \cdot 10^3$	$z \cdot 10^3$	Biso ( $\text{\AA}^2$ )
H(N1)	48(5)	180(5)	-7(2)	2.3(0.8)
H(C2)	291(5)	6(5)	30(2)	2.9(0.9)
H(C3)	529(5)	-108(6)	65(3)	3.9(1.1)
H(C5)	578(6)	-2(7)	279(3)	4.8(1.2)
H(C6)	316(5)	110(6)	242(3)	3.6(1.0)
H1(N4)	769(6)	-160(6)	169(3)	4.7(1.2)
H2(N4)	771(6)	-129(6)	245(3)	5.1(1.3)
H(C8)	379(5)	632(5)	98(3)	3.6(1.0)
H1(C11)	444(6)	714(6)	-110(3)	5.4(1.3)
H2(C11)	330(6)	710(6)	-182(3)	4.6(1.2)
H3(C11)	280(8)	787(8)	-122(4)	8.0(1.8)
H1(C12)	-65(8)	270(8)	-204(4)	8.6(1.9)
H2(C12)	33(11)	402(11)	-210(5)	12.4(2.7)
H3(C12)	-95(9)	375(9)	-165(4)	9.8(2.0)

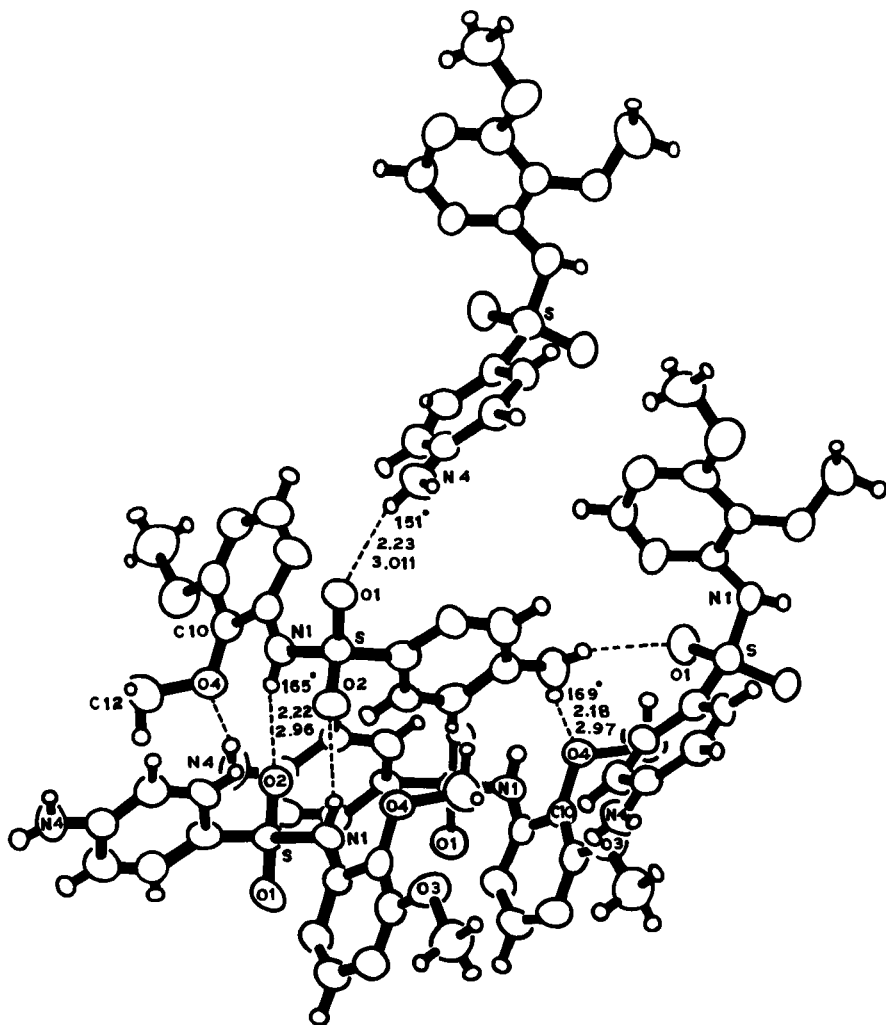


Figure 2 — Intermolecular hydrogen bonds observed in the sulfadoxine crystal.

bonds are  $43^\circ$ ,  $61^\circ$  and  $21^\circ$  for  $N1SC1C6[C2]$ ,  $C1SN1C7$ , and  $SN1C7N2[O3]$ , respectively. The dihedral angle between the pyrimidine and phenyl ring is  $98^\circ$ . The two methoxy groups of sulfadoxine are para and meta to the N2 position, but only the para

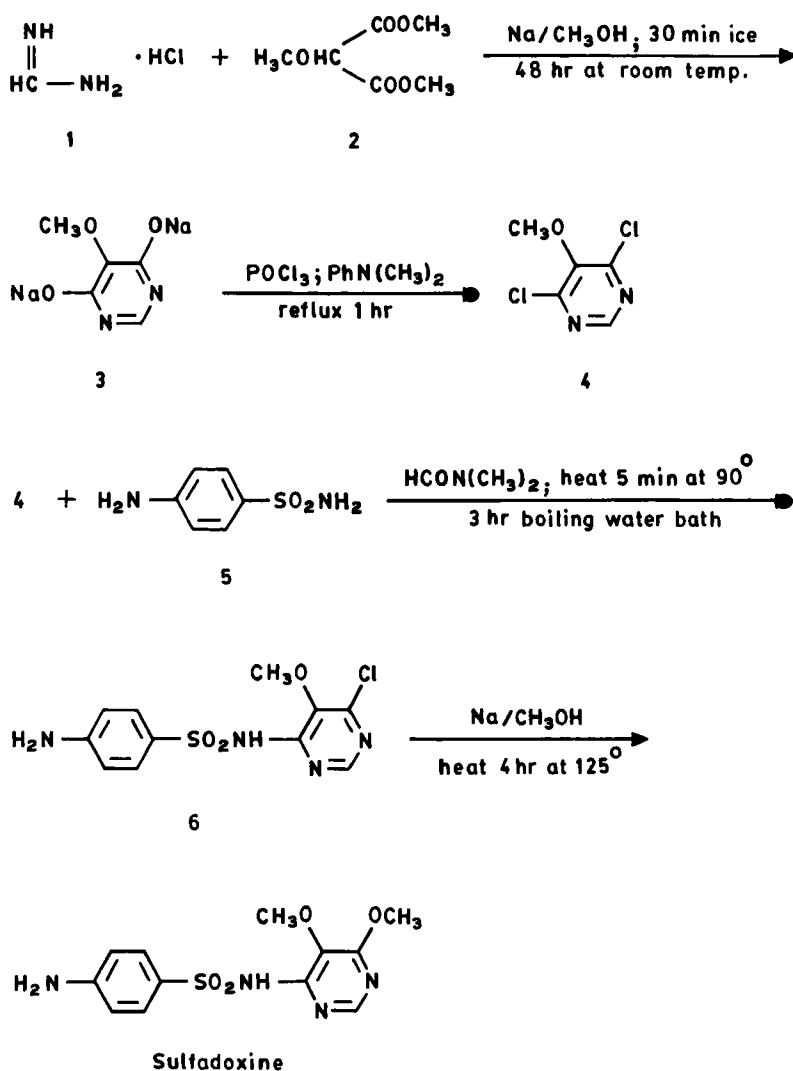
group can increase the basicity of N2 by resonance. Although the methyl of the para-methoxy group on C9 is 10° from coplanarity with the pyrimidine ring, the methyl of the meta-methoxy group is bent 52° out of the plane of the heterocyclic ring. Moreover, the O4-C10 bond distance of 1.370 Å is significantly longer (about 0.03Å) than the O3-C9 bond. It thus appears that the meta-methoxy group has less conjugation with the pyrimidine ring than the para one. The O4 atom of sulfadoxine is more basic than the other methoxy oxygen as evidenced by its participation in an intermolecular hydrogen bond (Figure 2). The Figure 2 also shows a pair of centrosymmetric hydrogen bonds between N1-H and O2. The ring nitrogen N2, however, is not involved in hydrogen bonding. The O1 of the sulfone moiety is hydrogen bonded to the proton on the anilino nitrogen N4 in the structure. The study carried out by Shefter *et al.*<sup>8</sup> suggests that the characteristics of certain hydrogen bond could possibly be important determinants for serum albumin binding of the sulfonamide. The serum albumin binding is one of the parameters which greatly influences the pharmacokinetics and activity of the sulfonamide. A more recent study on the crystal structure of sulfadoxine is carried out by Mazus *et al.*<sup>9</sup>

The X-ray crystallographic studies have also suggested that the amido tautomer of sulfadoxine (hydrogen atom attached to N1) is the stable form in the solid.<sup>8</sup> Identification of the tautomers (amido or imido) in the solid state and in solution of some sulfonamide derivatives by spectrometric methods is also reported.<sup>10</sup> Studies on the polymorphism and solid solution formation have been carried out.<sup>11</sup>

### 3. Synthesis

Sulfadoxine was synthesized in the laboratories of F. Hoffmann-La Roche & Co., Switzerland. Several methods of its synthesis are reported.<sup>12-15</sup> Scheme I outlines the synthesis<sup>13</sup> of sulfadoxine. The pyrimidine part of the molecule is prepared by reacting formamidine hydrochloride (1) in a solution of sodium in methanol with dimethyl  $\alpha$ -methoxymalonate (2) and keeping the mixture in

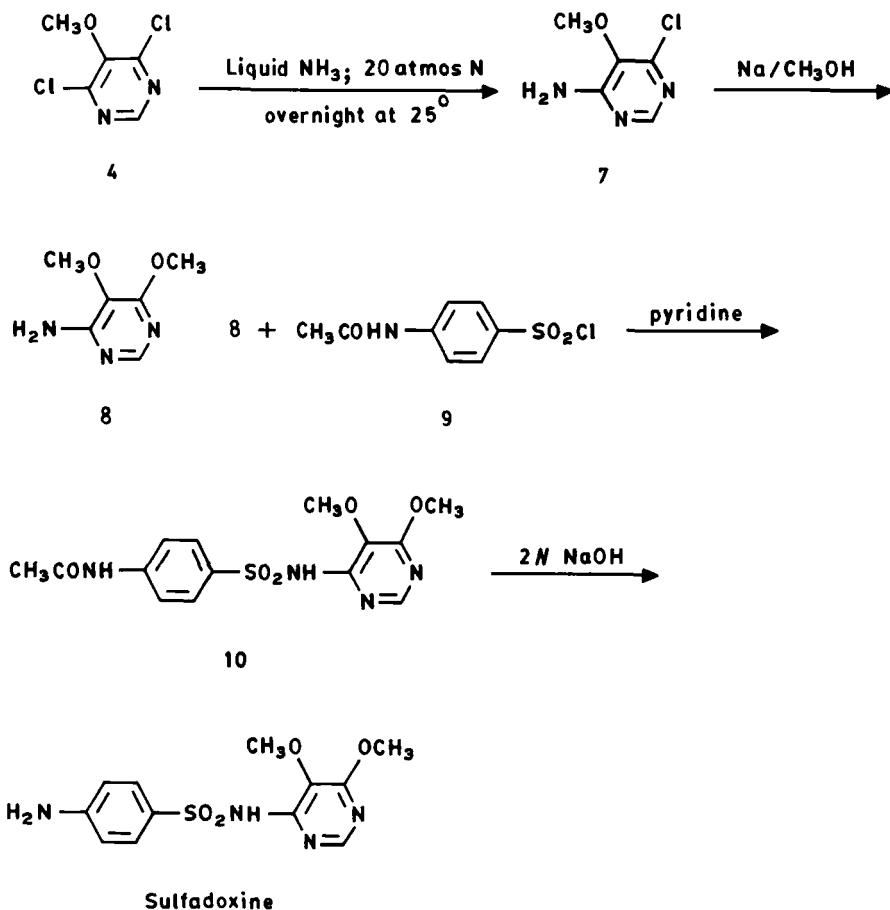




Scheme I — Synthesis of Sulfadoxine

ice for 30 minutes and at room temperature for 48 hours to give sodium salt of 4,6-dihydroxy-5-methoxypyrimidine (3). A mixture of (3),

phosphorus oxychloride and *N,N*-dimethylaniline is refluxed for 1 hour to give 4,6-dichloro-5-methoxypyrimidine (4). The latter is added at 90-100° to a mixture of sodium salt of *p*-aminobenzenesulfonamide (5) in dimethylformamide and heating first for five minutes at 90° followed by heating for 3 hours in a boiling water bath to give 4-sulfanilamido-5-methoxy-6-chloropyrimidine (6). Sulfadoxine is finally obtained by adding (6) to a solution of sodium in methanol and heating the mixture for 4 hours at 125°.



Scheme II - Alternate Synthetic Route to Sulfadoxine

An alternate route<sup>14</sup> to sulfadoxine, outlined in Scheme II, involves first preparing 4-amino-5-methoxy-6-chloropyrimidine (7) from 4,6-dichloro-5-methoxypyrimidine (4) by shaking the latter with liquid ammonia in an autoclave at 20 atmosphere nitrogen overnight at 25°. In the process ammonia is distilled off, the residue washed with water and dissolved in 2N hydrochloric acid, the solution treated with carbon, filtered and neutralized with sodium carbonate to give (7). The 4-aminopyrimidine (7) can also be prepared by passing dry ammonia into a solution of (4) in absolute dimethylformamide for 7 hours at 80°. Refluxing (7) with a solution of sodium in methanol for 18 hours gives 4-amino-5,6-dimethoxypyrimidine (8). Addition of p-acetamidobenzenesulfonyl chloride (9) to a solution of (8) in pyridine in such a way that the temperature does not rise above 2-3° followed by stirring for 16 hours at 1-2° gives 4-(N<sup>4</sup>-acetyl-sulfanilamido)-5,6-dimethoxypyrimidine (10). Hydrolysis of (10) by stirring in 2N sodium hydroxide for 1.5 hours gives sulfadoxine.

#### 4. Stability and Degradation

In general sulfadoxine is a stable compound. The USP directs that it should be preserved in well-closed, light resistant containers.<sup>4</sup> A solution of sulfadoxine adjusted to pH 10 with sodium hydroxide, may be sterilised by autoclaving in sealed containers in which the air has been replaced by nitrogen or other suitable gas.<sup>1</sup> Auterhoff and Schmidt<sup>16</sup> have described the pyrolysis and hydrolysis products of sulfadoxine. On pyrolysis it gives 4-amino-5,6-dimethoxypyrimidine and on acid hydrolysis the products obtained are 4-aminobenzenesulfonic acid, 4-amino-5,6-dimethoxypyrimidine and its mono demethylated analogue.

#### 5. Metabolism and Pharmacokinetics

Metabolism and pharmacokinetics of sulfadoxine and its combination with trimethoprim have been extensively studied.<sup>17-67</sup> Sulfadoxine is readily

absorbed from the gastrointestinal tract. The fate of sulfadoxine was investigated in rat, rabbit, monkey, cow and man; N<sup>4</sup>-acetyl derivative was found to be the major metabolite<sup>20,24,26,27</sup> (Figure 3). Sulfadoxine is very slowly excreted,

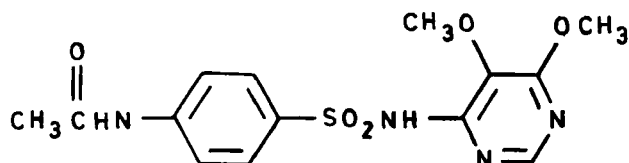


Figure 3 — Major Metabolite of Sulfadoxine

only about 8% being recovered from the urine in twentyfour hours and about 30% in seven days, up to 60% being excreted as the acetyl derivative and about 10% as the glucuronide. Sulfadoxine is a sulfonamide with a particularly long half-life of 7 to 9 days.<sup>68</sup> Blood concentrations of 80 to 120  $\mu\text{g}$  per ml were produced when sulfadoxine was given to children as a single dose of 25 mg per kg body-weight, the blood concentration fell to 20 to 60  $\mu\text{g}$  per ml after 7 days. If a daily maintenance dose of 5 mg per kg was also given blood concentrations were maintained at 80 to 90  $\mu\text{g}$  per ml. A single dose of 50 mg per kg produced a blood concentration of about 200  $\mu\text{g}$  per ml which did not fall below 60  $\mu\text{g}$  per ml after 7 days. Less than 10% of sulfadoxine was conjugated in the blood. About 30% of a single dose was excreted in the urine in 7 days, about 50% as the acetyl derivative and a small amount as glucuronide.<sup>22</sup> Blood concentrations of 130 to 200  $\mu\text{g}$  per ml were achieved in 31 patients who received sulfadoxine 1.5 to 2 g weekly, the average half-life was about 7 days.<sup>23</sup>

Recently, pharmacokinetics of sulfadoxine in combination with pyrimethamine (Fansidar) has been studied in healthy Japanese males.<sup>65</sup> The T<sub>max</sub> (time for maximum plasma concentration), C<sub>max</sub> (maximum plasma concentration) and T<sub>1/2</sub> values for sulfadoxine in Fansidar were found to be

3.6 hr, 124.6  $\mu\text{g/ml}$  and 182.4 hr, respectively. Ten days after Fansidar administration 7.5% of the administered sulfadoxine was excreted in the urine in its intact form; 16.7 and 11.6% of the sulfadoxine dose was excreted as acetylated sulfadoxine and sulfadoxine glucuronide, respectively. In another recent study<sup>66</sup> carried out in seven healthy volunteers after single oral dose of Fansidar a comparison was made between pharmacokinetics of sulfadoxine and pyrimethamine calculated from whole blood and plasma data. The mean whole blood to plasma concentration ratios of sulfadoxine and pyrimethamine were 0.62 and 0.87, respectively. The elimination half-lives of sulfadoxine and pyrimethamine were similar in whole blood and plasma. The apparent volume of distribution and clearance of both the drugs in whole blood were significantly higher than the corresponding plasma values. Pharmacokinetic studies of the antimalarial drug Fansimef which contains pyrimethamine, sulfadoxine and mefloquine were carried out recently in Brazilian volunteers.<sup>67</sup> After the last maintenance dose the following kinetic parameters were determined for pyrimethamine, sulfadoxine and mefloquine, respectively: elimination half-life = 123, 179 and 550 hr; volume of distribution in the post distribution phase = 2.5, 0.15 and 18.6 l/kg; and total systemic clearance = 14.0, 0.64 and 24.0 ml/hr/kg.

## 6. Protein Binding

Binding to human plasma proteins is an important factor in determining the excretion rate of sulfonamides. Protein binding studies of sulfadoxine have been carried out.<sup>69-73</sup> About 88-90% of the drug is reported to be bound to serum proteins of cows, rabbits and humans.<sup>72</sup> Walker<sup>71</sup> has shown that at concentrations of 0.4 mM, 0.8 mM and 1.2 mM sulfadoxine is bound 96%, 88% and 79%, respectively. The binding capacity of the plasma albumin ( $r_{\text{max}}$ ) at 1.6 mM concentration of sulfadoxine is 1.91 with two binding sites. The value of K, the dissociation constant of the sulfadoxine-albumin complex, has

been found to be 0.08. Pharmacokinetic models have been developed taking into account the protein binding which define the relation between renal or total clearance and the slope of the time-log concentration curve in plasma and plasma water.<sup>70</sup> In a recent study an equation based on regular solution theory was used to relate the solubility parameter to the binding of sulfonamide by plasma proteins.<sup>73</sup>

## 7. Toxicity

A severe form of erythema multiforme, associated with widespread lesions of the skin and mucous membranes (termed as Stevens-Johnson syndrome) and epidermal necrolysis (Lyell's syndrome) have been reported following treatment with sulfadoxine.<sup>1</sup> A recent study has indicated that sulfadoxine in combination with pyrimethamine does not affect several blood serum indexes of liver dysfunction e.g. alkaline phosphatase, glutamate oxaloacetate transaminase, total and fractional proteins, suggesting that the combination is not hepatotoxic.<sup>74</sup>

## 8. Methods of Analysis

### 8.1 Elemental Composition

The elemental composition of sulfadoxine is as follows<sup>75</sup>:

<u>Element</u>	<u>Per cent</u>
C	46.44
H	4.55
N	18.05
O	20.62
S	10.33

## 8.2 Identification Color Tests

Sulfadoxine gives an orange red precipitate when 100 mg of it dissolved in 2 ml of 2N hydrochloric acid are treated with 0.2 ml of sodium nitrite solution followed after one to two minutes by the addition of 1 ml of  $\beta$ -naphthol solution.<sup>3</sup> The reaction is due to the primary aromatic amino group present in sulfadoxine. The other colour tests described are with coniferyl alcohol - orange; copper sulfate - green; mercurous nitrate - black; and with nitrous acid - yellow.<sup>2</sup>

## 8.3 Titrimetric Analysis

Nitrite titration is the method of choice to assay sulfadoxine.<sup>4</sup> The method involved is as follows. Weigh accurately about 500 mg and transfer to a suitable open vessel. Add 20 ml of hydrochloric acid and 50 ml of water, stir until dissolved. Cool to about 15°, and slowly titrate with 0.1M sodium nitrite solution that has been previously standardized against USP sulfanilamide. Determine the end-point electrometrically, using suitable electrodes (platinum-calomel or platinum-platinum). Place the burette tip below the surface of the solution to eliminate air-oxidation of the sodium nitrite, and stir the solution gently, using a magnetic stirrer, without pulling a vortex of air under the surface, maintaining the temperature at about 15°. When the titration is within 1 ml of the end-point, add the titrant in 0.1 ml portions, allowing not less than 1 minute between additions. Each ml of 0.1M sodium nitrite is equivalent to 31.03 mg of  $C_{12}H_{14}N_4O_4S$ .

A complexometric titration for the determination of sulfadoxine in pharmaceutical preparations has been described.<sup>76</sup> It involves precipitation of the drug by adding a known amount of copper ions (0.1M  $CuSO_4$ ) at pH 6 and titrating the excess of  $CuSO_4$  with 0.02M EDTA solution after addition of ethanol and an indicator.

#### 8.4 Bioassay

Several microbiological methods for the determination of sulfadoxine residues in edible tissues and milk have been reported.<sup>77-85</sup> In order to facilitate the detection of the sulfonamide the growth medium is supplemented with trimethoprim exploiting the synergistic effect between trimethoprim and the sulfonamide. The standardized conditions include the use of a sporulating organism, Bacillus subtilis, an inoculum size of  $0.5 \times 10^5$  spores/ml medium and 5 ml of medium (pH 6.0) per plate.<sup>81</sup> A pre-incubation diffusion time of 1 hour at room temperature is recommended before incubation. In an improved method of bioassay described by Nouws et al.<sup>84</sup> the detection limit for sulfadoxine residues in urine, milk, kidney and muscle ranged from 0.05 to 0.2  $\mu\text{g/ml}$ . Other micro-organisms employed in the bioassay studies are Bacillus megaterium<sup>77</sup>, Escherichia coli<sup>79,80</sup> and Sarcinia lutea.<sup>77</sup>

In a recent study in vitro tests for Plasmodium falciparum sensitivity to sulfadoxine and pyrimethamine and both drugs in combination were performed in four kinds of culture media each differing in p-aminobenzoic acid and folic acid concentrations.<sup>85</sup> The optimal concentrations of p-aminobenzoic acid and folic acid for parasite growth and drug susceptibility as evaluated by microscopic examination and by the extent of incorporation of radioactive [ $^{14}\text{C}$ ]pyrimethamine and [ $^{14}\text{C}$ ]sulfadoxine were 10 ng/ml and 2 ng/ml, respectively.

#### 8.5 Colorimetric Analysis

A colorimetric method for analysis of sulfadoxine based on formation of a colored indophenolic derivative as described by Vignoli et al.<sup>86</sup> is as follows. Place a 2 ml (0.2 mg) sample of the sulfonamide solution (0.1/1000) in a tube with a ground stopper, add 0.5 ml 0.1N HCl and 0.25 ml 25% aqueous phenol solution, place in a boiling water bath for 5 minutes, add 3 ml chloramineT solution with 5% chlorine,



dilute to 10 ml with distilled water. Place in water bath for an additional 10 minutes, cool and extract the color with 10 ml isoamyl alcohol. A continuous procedure adapted to the auto-analyser is given which is based on the colorimetric determination at 540 nm of an azo dye formed by coupling the sulfonamide with  $\alpha$ -naphthylethylenediamine.<sup>87</sup> Colorimetric determination involving the use of Z- $\alpha,\beta$ -dinitrostilbene as a reagent is also described.<sup>88</sup>

## 8.6 Spectrophotometric Analysis

### 8.61 Ultraviolet

Sulfadoxine can be determined by ultraviolet spectrophotometry in pharmaceutical formulations and in biological fluids. It has been determined in Fansidar tablets<sup>89</sup> and Fansidar syrup<sup>90</sup> spectrophotometrically at 220-320 nm. The estimation in the syrup involves extraction with diethyl ether-acetone mixture, evaporating the solvent mixture, drying the resulting powder, dissolution in 0.1N HCl and 0.1N NaOH solutions and determination of absorption spectra. Sulfadoxine has been determined in the presence of chloroquine, quinine and pyrimethamine in urine and blood.<sup>91</sup> Chloroquine, quinine and pyrimethamine were extracted together with diethyl ether from urine or blood (the latter heat treated to break drug-protein bonds) at pH 12.5. The residual urine and blood were brought to pH 4.1 and sulfadoxine was extracted with ethylene dichloride and back-extracted into 0.1N sulphuric acid. It was determined directly in the acid extract by ultraviolet spectrophotometry at 265 nm. Extraction recoveries of sulfadoxine from urine and blood were 73 and 61%, respectively. Limits of detection of sulfadoxine in urine and blood were 58 and 126 ng/ml, respectively. The procedure is sufficiently sensitive and reproducible to monitor therapeutic concentrations of these antimalarials.

The effects of substituents and solvents on the E, K and B UV absorption bands of different sulfonamides have been studied.<sup>92,93</sup> Extinction coefficients values can be used to determine

sulfonamides in medicinal preparations.

### 8.62 Infrared

Trius<sup>94</sup> has discussed the use of infrared spectroscopy in qualitative analysis of sulfadoxine.

### 8.7 Mass Spectrometric Analysis

Mass spectrum of sulfadoxine and its comparison with spectra of N-methylated analogue fixed in the amino or imino form indicated that sulfadoxine exists in the amino form.<sup>95</sup> The electron impact mass spectra (Figure 4) of sulfadoxine was measured in GC-MS mode under conditions generally used in residue analysis.<sup>96</sup> The molecular ion peak was not observed and a strong peak, the base peak, was observed at  $m/z$  245. The characteristic selected ions allowed an unequivocal identification of the sulfonamide in

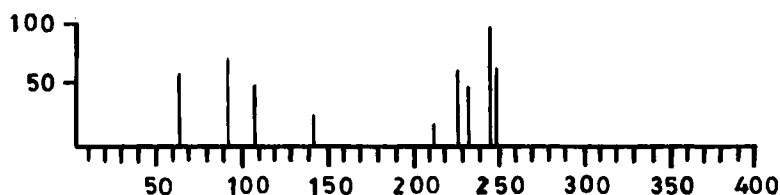


Figure 4 — Mass Spectrum of Sulfadoxine

residue analysis. Tandem mass spectrometry is shown to be a powerful method for the trace analysis of sulfonamides not only for confirmatory analysis but more importantly for rapid screening and quantification. Recently, collisionally activated dissociation mass spectra, observed with a hybrid tandem instrument, of the chemical ionization protonated molecular ions of five sulfonamides including sulfadoxine have been used as the basis of a rapid screening procedure for these drugs in crude extracts of pig's kidney by scanning to detect the parents of a characteristic

daughter fragment.<sup>97</sup> Extracts were introduced without chromatography by a moving belt interface. Detection limits of 0.1 mg/kg were achieved. Confirmation was made by obtaining daughter ion spectra of the protonated molecular ions. Multiple reaction monitoring with a stable isotope analogue as internal standard permitted the quantification of targeted compounds with high sensitivity and precision. In another study<sup>98</sup> the use of collision-induced dissociation mass analysed ion kinetic energy spectrometry (CID/MIKES) has demonstrated that direct confirmation of sulfonamides can be achieved without chromatography for extracts of pig liver that have received some degree of clean-up.

## 8.8 Chromatographic Analysis

### 8.81 Paper Chromatography

The following system for paper chromatography of sulfadoxine has been described.<sup>99</sup>

Paper:	Whatman No.1, sheet 14 x 6 in, buffered by dipping in a 5% solution of sodium dihydrogen citrate, blotting and drying at 25° for 1 hour.
Solvent:	4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of <u>n</u> -butanol
Equilibration:	None
Development:	Ascending, in a tank 8 x 11 x 15.5 in. Time of run, 5 hours
Location:	a. Examination under ultraviolet light b. By diazotisation followed by alkaline $\beta$ -naphthol spray
R <sub>f</sub> :	0.86

### 8.82 Thin-Layer Chromatography

The following thin-layer chromatographic systems have been recommended for the identification of sulfadoxine.<sup>2,100</sup>

<u>Solvent System</u>	<u>Plate</u>	<u>R<sub>f</sub> Value x 100</u>
Methanol-Strong ammonia solution (100:1.5)	Silica gel G, 250 $\mu$ m thick, dipped in or sprayed with 0.1M KOH in methanol	67
Chloroform-Acetone (4:1)	Silica gel 250 $\mu$ m thick	37
Ethyl acetate-Methanol-Strong ammonia solution (85:10:5)	Silica gel 250 $\mu$ m thick	08
Ethyl acetate	Silica gel 250 $\mu$ m thick	51
Chloroform-Methanol (4:1)	Silica gel 250 $\mu$ m thick mixed with 0.1M NaOH	69
Chloroform-Carbon tetrachloride-Methanol (7:2:1)	Silica gel 250 $\mu$ m thick mixed with 0.1M KHSO <sub>4</sub>	50
Ethyl acetate-Methanol (9:1)	Silica gel 250 $\mu$ m thick neutral	69
Acetone-Methanol (4:1)	Silica gel 250 $\mu$ m thick mixed with 0.1M NaOH	73

Other solvent systems described are:

<u>Solvent System</u>	<u>Reference</u>
Chloroform-Methanol-Dimethylformamide (20:2:1)	4
Chloroform-Methanol (10:1)	101
Chloroform-Butanol-Acetone-85% Formic acid (80:20:20:20)	101
Chloroform-Methanol-Butanol-2% Ammonium hydroxide (80:10:10:1)	101
Chloroform-Butanol (4:1)	102
Heptane-Chloroform-1 in 20 Solution of methanol in alcohol-Glacial acetic acid (4:4:4:1)	4

Detection of spot of sulfadoxine on the plate has been carried out by different methods such as visualization under ultraviolet light<sup>4</sup>, diazotisation followed by alkaline  $\beta$ -naphthol spray<sup>99</sup>, diazotisation followed by spray of an alcoholic solution of 1-(1-naphthyl)ethane-1,2-diammonium dichloride<sup>4</sup>, spraying with fluorecamine solution<sup>102</sup>, exposure to iodine vapours<sup>101</sup> or by alkaline copper sulfate.<sup>100,101</sup> Recently the use of  $\pi$ -acceptors such as 2,5-dichloro-p-benzoquinone in dimethyl sulfoxide as spray reagents for the visual detection of the sulfonamides has been described.<sup>103</sup>

A rapid thin-layer chromatographic procedure for the detection of sulfadoxine in animal tissues such as muscle and kidney of swine, chicken and cattle has been described.<sup>102,104</sup> The method consists of an extraction with dichloromethane, cleaning up of the extracts on a sep-Pak silica disposable column, and analysis by thin-layer chromatography on a silica plate

with chloroform-butanol (4:1) as the system. The  $R_f$  value of sulfadoxine in the system is 0.65. In spiked tissue the presence of the sulfonamide at concentrations of 0.05 mg/kg and higher is easily demonstrated. Direct detection of sulfadoxine in tissue extracts of kidney and lean tissue under the same chromatographic conditions is also described<sup>102</sup>. Analysis of sulfadoxine residues in kidneys and muscles of slaughtered animals by thin-layer chromatography and its comparison with microbiological analysis has been done<sup>82</sup>. A combined microbiological and thin-layer chromatographic method for the identification of sulfonamides in animal tissues has also been reported.<sup>105</sup>

Recently high performance thin-layer chromatography (HPTLC) technique has been developed for rapid identification and quantification of sulfonamides.<sup>106,107</sup> A TLC screening method with 0.067M potassium dihydrogen phosphate - 0.067M disodium hydrogen phosphate-methanol (390:10:80) as solvent and detection at 260 nm on high-performance plates for differentiation and detection of 23 different sulfonamides in muscle, kidney and serum of slaughtered animals has been developed by Schlatterer.<sup>106</sup> The sulfonamides were derivatized after chromatography on plates by spraying with a solution of fluorescamine and amounts of individual sulfonamide present were quantified by measurement of fluorescence remission. A number of developing solvents for application either in one-dimensional or two-dimensional chromatography mode of the linear developing chamber is proposed. Knupp et al.<sup>107</sup> have estimated  $R_f$  values of twenty sulfonamides including sulfadoxine in seven solvent systems by an improved HPTLC method. Based on these data a simple identification scheme has been developed demanding only three solvent systems for complete separation. Quantification of corresponding fluorescamine derivatives at nanogram level has been performed by the use of a TLC-scanner configured online with a microcomputer. The  $R_f$  values of sulfadoxine in different solvent systems on precoated silica gel 60 HPTLC plates have been given as follows:

<u>Solvent System</u>	<u>R<sub>f</sub> Value</u>
Chloroform-Butanol- Petroleum ether (40-60°) (1:1:1)	0.74
Ethyl acetate-Methanol- Ammonia (25%) (30:15:1)	0.41
Dichloromethane-Methanol (95:5)	0.63
Acetonitrile-Chloroform- Ammonia (25%) (35:10:0.2)	0.48
Diethyl ether	0.21

#### 8.83 Gas Chromatography

Bonini et al.<sup>108</sup> reported a gas chromatographic method for the determination of sulfadoxine in blood and urine using a column packed with 2% OV-17 on Chromosorb W AW DMCS 100-120 mesh with nitrogen as the carrier gas and the column temperature programmed to increase from 250-350° at 8° per minute. The limit of detection was 2.31 ng. The recoveries in blood and urine were 70.2 and 82.4%, respectively, for 10 µg sulfadoxine per ml. A procedure is described for the extraction, clean-up, derivatization and quantitative determination of sulfadoxine residues in meat, milk and eggs.<sup>109</sup>

#### 8.84 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is one of the major analytical techniques for the determination of sulfadoxine.<sup>110,111</sup> Cobb and Hill<sup>110</sup> have described a HPLC method on a 250 mm long stainless-steel column with an internal diameter of 4 mm packed with Spherisorb S5W 5-µm-diameter spherical silica gel particles using cyclohexane-ethanol-acetic acid (85.7:11.4:2.9) as the mobile phase. The chromatograph was operated at ambient temperature and the

mobile phase was driven at a rate of 2 ml/min. Elution was monitored at 250 nm using a variable wavelength spectrophotometer. Retention time (sec) of 474 is given for sulfadoxine. A HPLC method for simultaneously determining sulfadoxine, its major metabolite N<sup>4</sup>-acetylsulfadoxine and pyrimethamine in human plasma has been described.<sup>112</sup> Plasma samples were extracted with ethylene dichloride and quinine was used as the internal standard. Reversed phase ion-pair chromatography was used to separate the compounds and the eluant was monitored by UV absorbance (254 nm). The column was 30 cm x 3.9 mm I.D., particle size 10  $\mu$ m,  $\mu$ Bondapak C<sub>18</sub>, and the mobile phase consisted of methanol-acetonitrile and water (25:15:60) containing 0.005M 1-pentane sulfonic acid. It was pumped at a flow-rate of 1.5 ml/min at ambient temperature. Retention times for sulfadoxine, N<sup>4</sup>-acetylsulfadoxine, quinine and pyrimethamine were 3.8, 4.7, 7.4 and 9.7, respectively. Another method for simultaneous determination of sulfadoxine and pyrimethamine in human plasma by reversed-phase HPLC describes use of Nucleosil C-18 as the support and acetonitrile-phosphate buffer pH 5.5 (28:72) as the mobile phase with tetrabutylammonium as counter ion.<sup>113</sup> The method is suitable for simultaneous determination of both the drugs after therapeutic doses of Fansidar. Bergqvist *et al.*<sup>114</sup> have recently described a HPLC method for determination of sulfadoxine in whole blood by finger prick and dried on filter paper. The technique was validated by comparing the sulfadoxine concentrations of simultaneously collected capillary blood dried on filter paper and conventional venous whole blood samples. Agreement between capillary blood dried on filter paper and venous whole blood was satisfactory. Limits of detection using 100  $\mu$ l of whole blood was found to be 25  $\mu$ mol/l (7.8  $\mu$ g/ml). Sulfadoxine was stable in the dried filter papers for at least 15 weeks at +37°. The concentration ratio between plasma and whole blood of sulfadoxine was found to be about 1.8. The sampling technique on filter paper offers a convenient method for overcoming many of the practical problems of blood sampling in the field.



HPLC has also been used to determine sulfadoxine residues in animal tissues.<sup>109,115</sup> A method for the determination in swine tissue involves cleaning of the tissue extract by passing through a cation exchange column followed by the HPLC analysis of the separated sulfonamide on a Chromopack column (250 x 4 mm) with acetonitrile-10 mM ammonium acetate pH 4.6 (3:7) as mobile phase and measured at 254 nm.<sup>115</sup>

Methods for HPLC analysis of sulfadoxine in combination with pyrimethamine<sup>4,116</sup>, and with trimethoprim<sup>117</sup> in pharmaceutical products are also described.

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## **SULPIRIDE**

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SULPIRIDE

## 1. Description

- 1.1 Nomenclature
  - 1.1.1 Chemical Names
  - 1.1.2 Generic Names
  - 1.1.3 Trade Names
  - 1.1.4 Chemical Abstract Registry Numbers
- 1.2 Formulae
  - 1.2.1 Empirical
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  - 1.2.3 Structural
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1. DESCRIPTION1.1 Nomenclature1.1.1 Chemical Names

(R,S)-5-(aminosulfonyl)-N-[ (1-ethyl-2-pyrrolidinyl) methyl]-2-methoxybenzamide

(R,S)-N-[ (1-ethyl-2-pirrolidinyl) methyl]-2-methoxy-5-sulfamoylbenzamide

(R,S)-N-[ (1-ethyl-2-pirrolidinyl) methyl]-5-sulfamoyl-o-anisamide

1.1.2 Generic Names

Sulpiride	USAN, BAN, DCF
Sulpiridum	NEN

1.1.3 Trade Names

Dixibon	(Wander)
Dobren	(Ravizza)
Dogmatil	(Delagrangé)
Dolmatil	(Squibb)
Equilid	(Lepetit)
Miradol	(Mitsui)
Mirbanil	(Boehringer Ingelheim)
Pyrikorpp 2	(Isei)
Levopraid	(Ravizza)

1.1.4 Chemical Abstract Registry Numbers

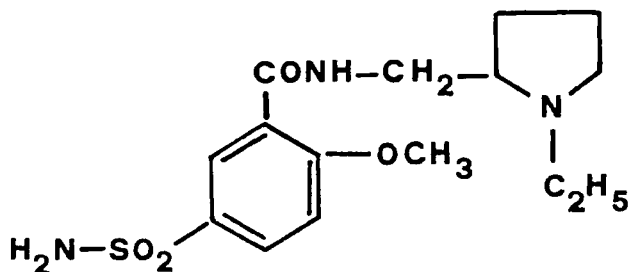
[15676-16-1]	sulpiride
[23672-06-2]	( $\pm$ )
[23694-14-6]	monohydrochloride
[54289-23-5]	sulfate
[53803-79-5]	sulfate (1:1)
[69907-66-0]	monohydriodide
[23756-79-8]	(R)-base
[23672-07-3]	(S)-base
[77111-58-1]	(S)-monohydrochloride

1.2 Formulae1.2.1 Empirical

$C_{15}H_{23}N_3O_4S$	Base
$C_{25}H_{23}N_3O_4S \cdot HCl$	Monohydrochloride
$C_{15}H_{23}N_3O_4S \cdot H_2SO_4$	Sulfate

1.2.2 Molecular weight

Base :	341.43
Monohydrochloride:	377.89
Sulfate:	439.31

1.2.3 Structural1.3 Elemental Composition

C, 52.77%; H, 6.79%; N, 13.31%; O, 18.74%;  
S, 9.39%.

1.4 Appearance

White, odorless, crystalline powder

## 2. PHYSICAL PROPERTIES

### 2.1 Infrared Spectrum

The infrared spectrum shown in fig. 1 was recorded with a Perkin Elmer 1310 spectrometer.

The spectrum of sulpiride was obtained from a potassium bromide dispersion.

The interpretation of the main absorption bands is given in table 1.

Table 1  
Infrared spectrum of sulpiride

IR Absorption band $\text{cm}^{-1}$	Assignments
3380	N-H of the sulfonamide group
3220-3160	N-H of the amide group
3080	N-H of aromatic
2990-2820	C-H of methylene and methyl group
1645	C=O of the amide group
1595	skeletal stretching of benzene ring
1550	N-H
1350	SO <sub>2</sub>
1185 and 1160	C-O of methoxy group
840	C-H out of plane of benzene ring

### 2.2 Nuclear Magnetic Resonance Spectra

#### 2.2.1 <sup>1</sup>H NMR

The proton magnetic resonance spectrum of sulpiride, shown in fig. 2, was obtained on a Varian X L 200 NMR spectrometer in DMSO-d<sub>6</sub> solution. The interpretation of the spectrum is given in Fig. 3

#### 2.2.2 <sup>13</sup>C NMR

The <sup>13</sup>C NMR spectrum of sulpiride obtained in DMSO-d<sub>6</sub> is shown in fig. 4. The spectrum was recorded on a Varian X L 200 NMR spectrometer at 50 MHz.

The assignments (1) are given in table 2.



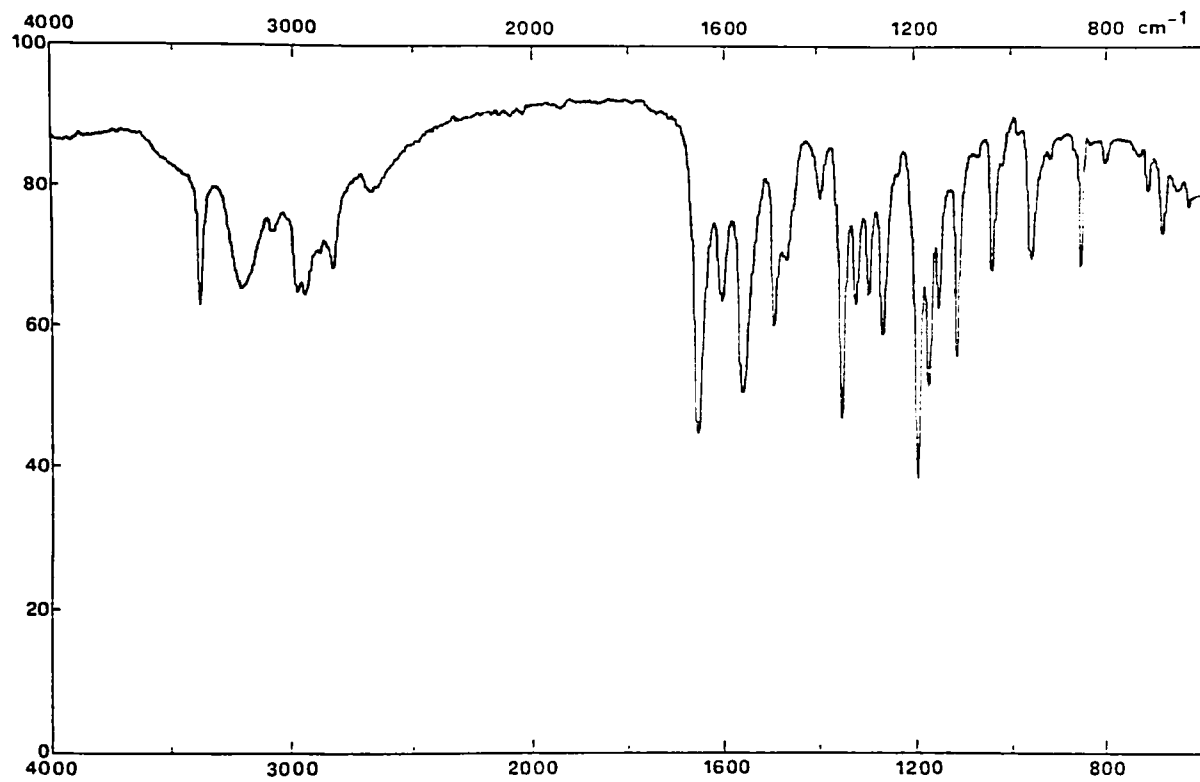


Fig. 1. - Infrared Spectrum of Sulpiride as KBr Disc

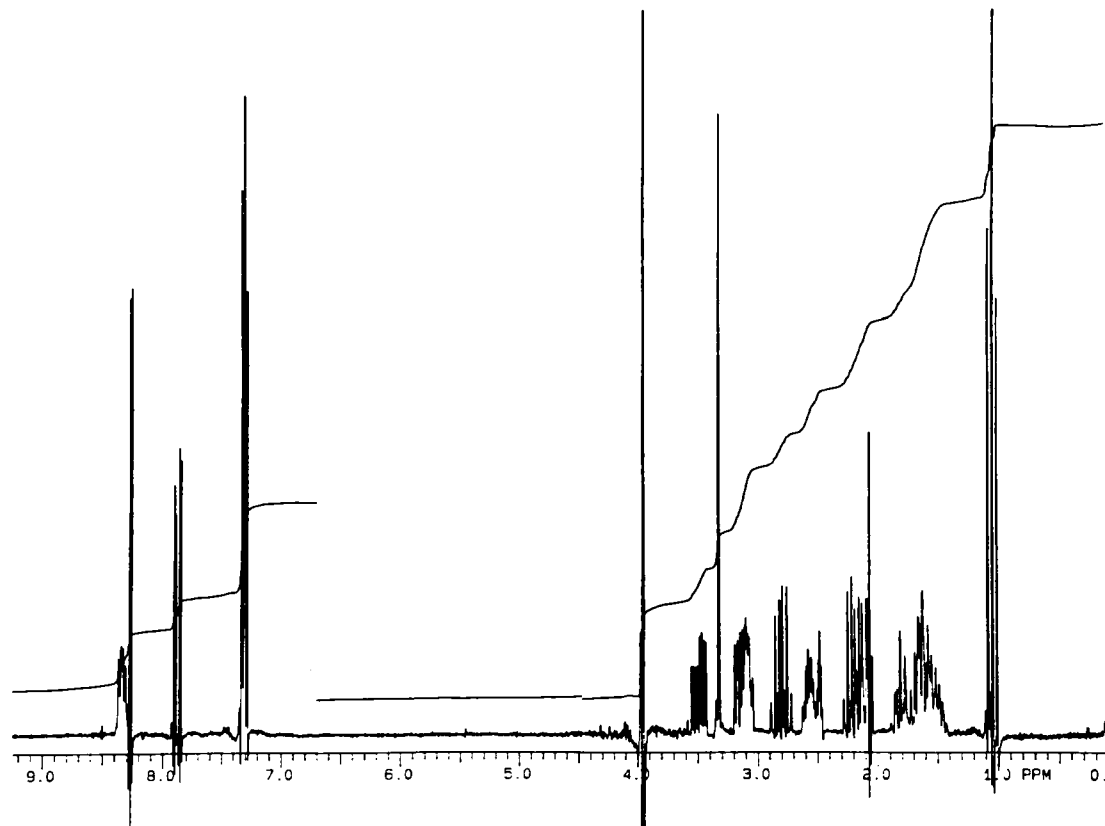


Fig. 2. - Proton NMR spectrum of sulpiride in DMSO-d<sub>6</sub>

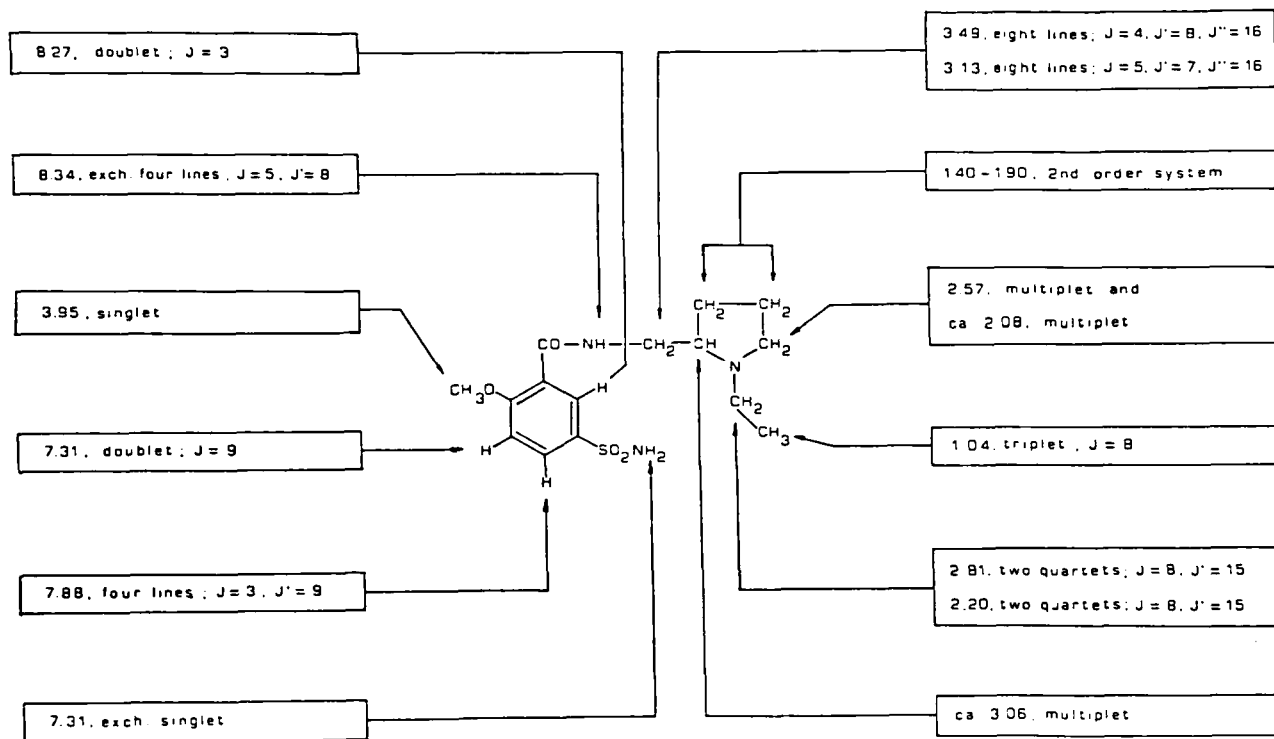


Fig. 3. - Interpretation of proton NMR spectrum of sulpiride

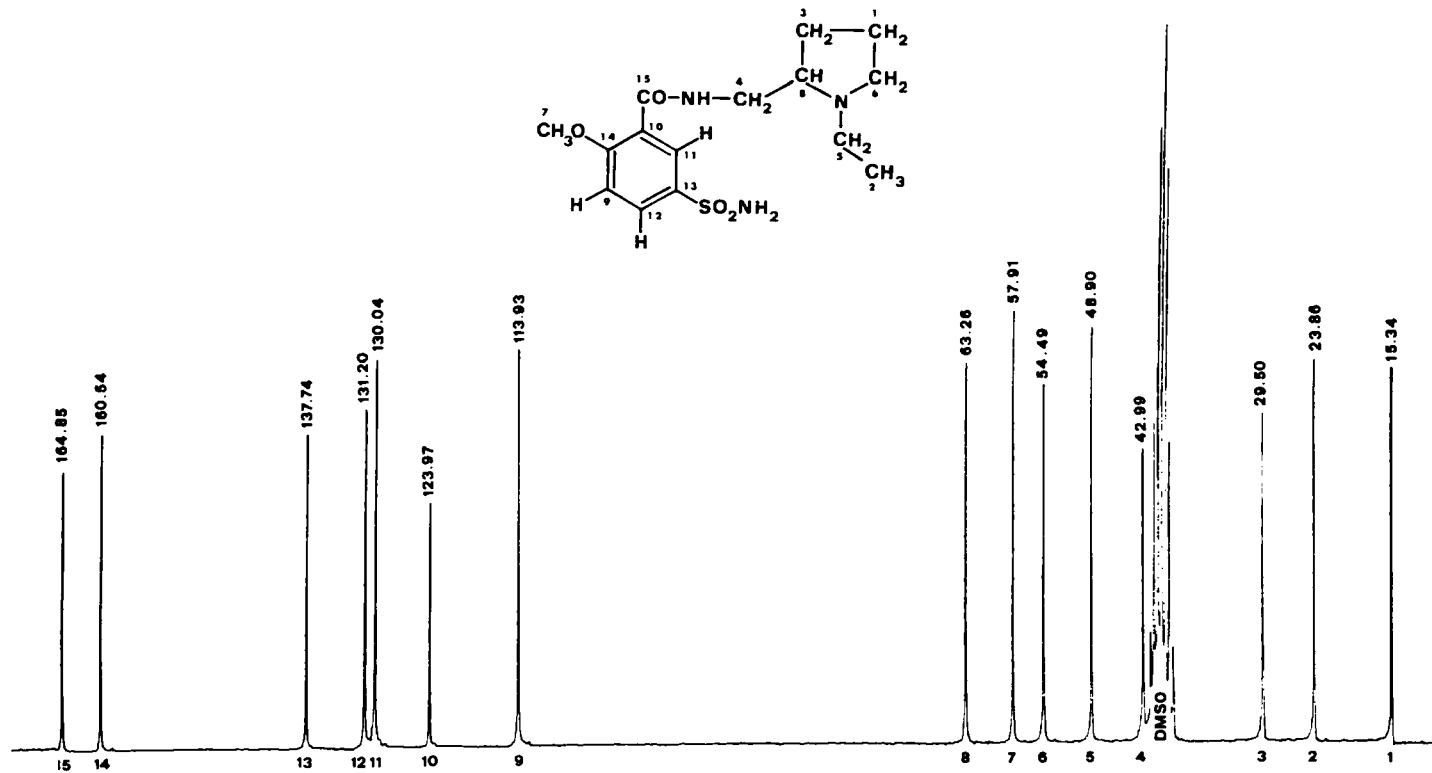
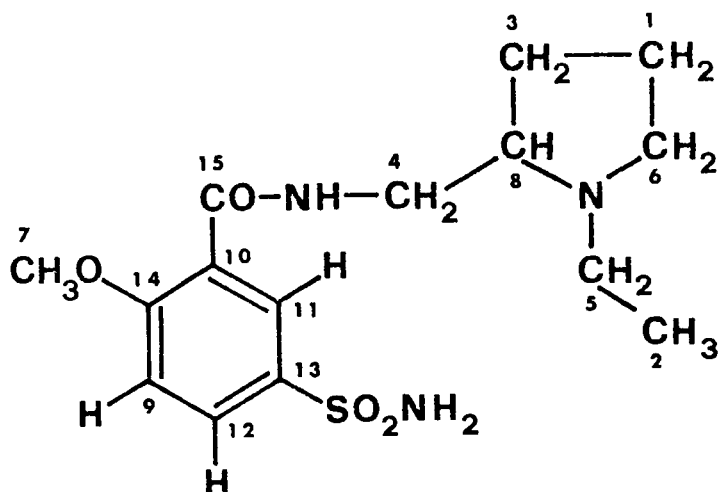


Fig. 4. - <sup>13</sup>C NMR spectrum of sulpiride in DMSO-d<sub>6</sub>

Table 2



Carbon position	Chemical shift TMS (ppm)	Multiplicity
1	15.34	Triplet
2	23.86	Quartet
3	29.50	Triplet
4	42.99	Triplet
5	48.90	Triplet
6	54.49	Triplet
7	57.91	Quartet
8	63.26	Doublet
9	113.93	Doublet
10	123.97	Singlet
11	130.04	Doublet
12	131.20	Doublet
13	137.74	Singlet
14	160.54	Singlet
15	164.85	Singlet

### 2.3 Mass Spectrum

The mass spectrum of sulpiride obtained by electron-impact ionization (fig. 5) was recorded on a Finnigan model 1020 mass spectrometer at 70 e.v.

The fragmentation pattern (2) is presented in scheme 1.

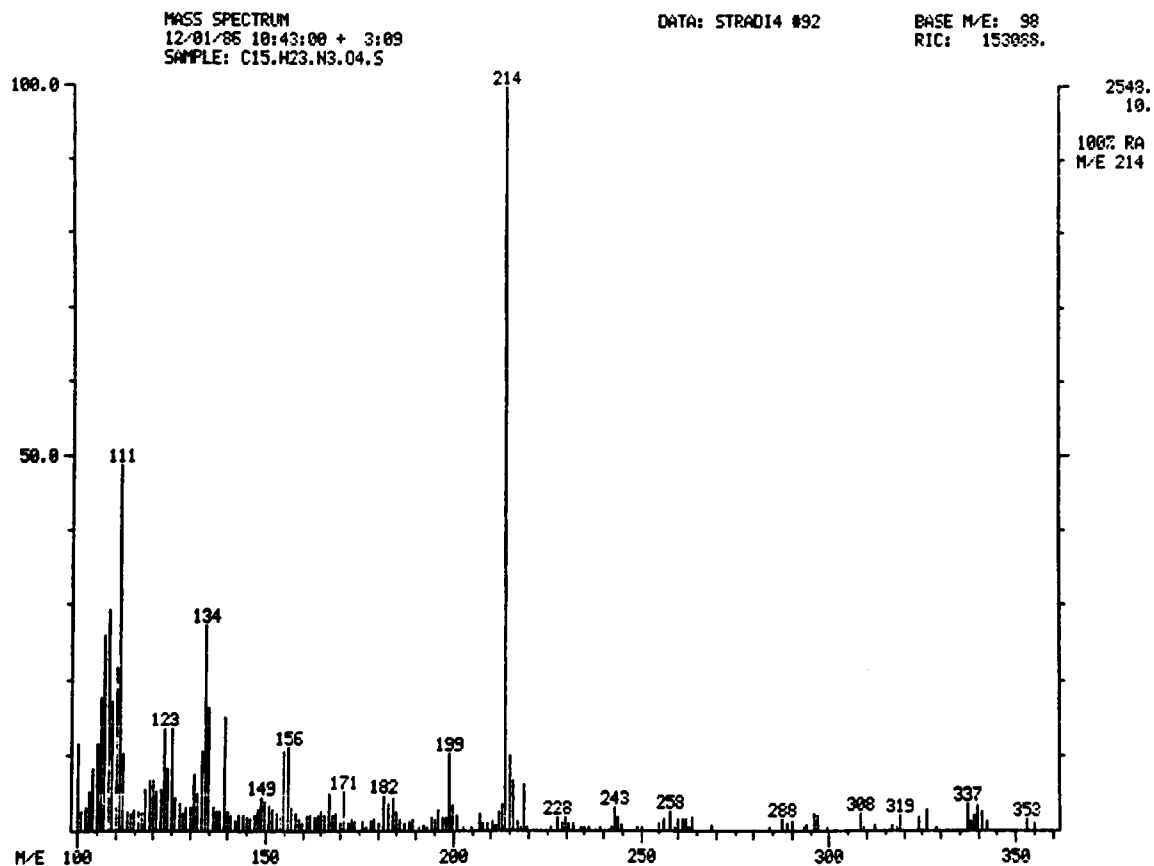
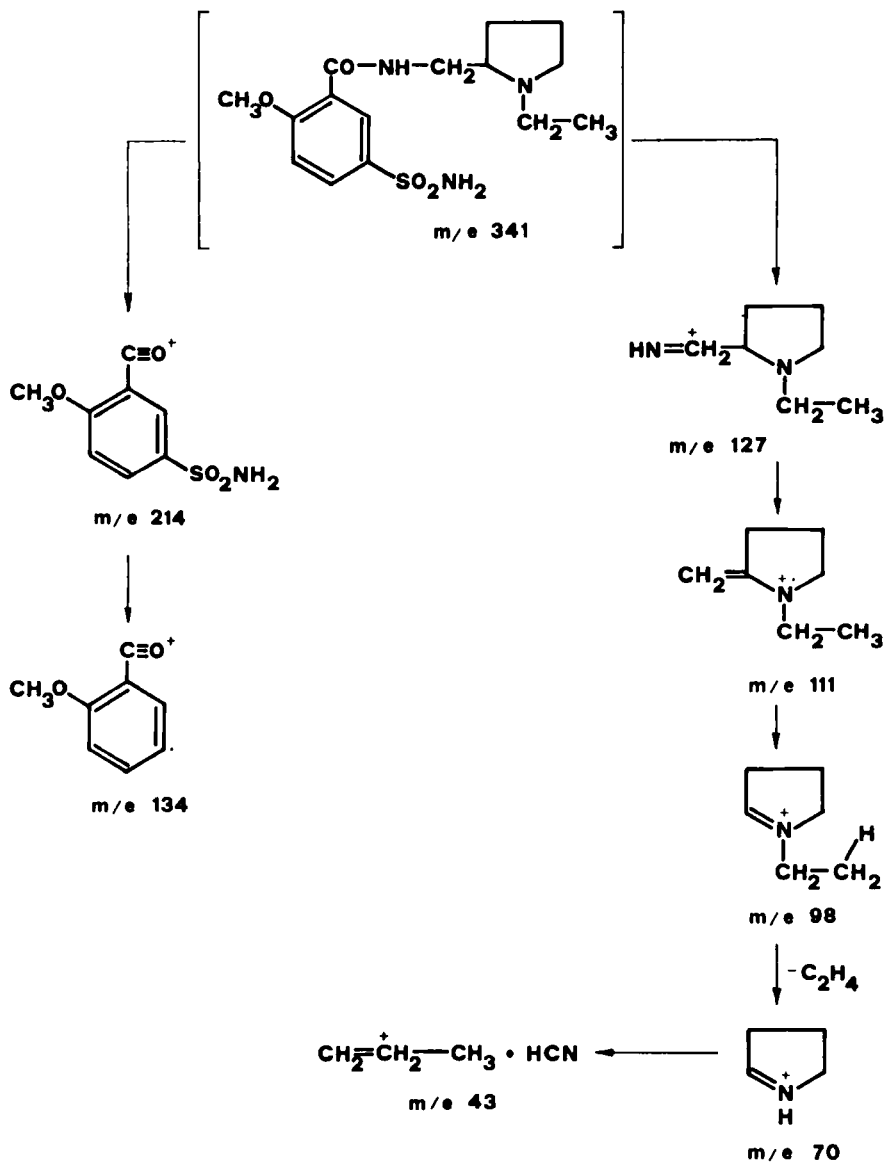


Fig. 5. - Mass spectrum of sulpiride by electron-impact ionization

### Fragmentation pattern for sulpiride

### SCHEME 1



## 2.4 Ultraviolet Spectrum

The ultraviolet absorption spectrum of sulpiride in methanol (1.02 mcg/ml) was obtained on a C.Erba Spectracomp. 601. The spectrum given in fig. 6 presents two maxima at 239 and 296 nm.

## 2.5 Fluorescence Spectrum

A solution of sulpiride in N HCl becomes fluorescent when excited in ultraviolet light. (3)

The excitation spectra (247, 291 and 344 nm) and emission spectra (388 nm) obtained using a Perkin Elmer MPF 44A spectrofluorometer on a solution of sulpiride (1.07 mcg/ml) in N HCl are provided in fig. 7 and 8.

## 2.6 Differential Scanning Calorimetry

Sulpiride and the S(-) form show a sharp endotherm in their DSC-thermograms. The data, reported in table 3 were determined with a DSC-2 Perkin Elmer apparatus. (4)

Table 3

<u>Form</u>	<u>Mp/K°</u>	<u>H (KJ-mol<sup>-1</sup>)</u>	<u>Purity</u>
(+)	450.7	46.13	99.70
(S)-(-)	459.3	42.02	99.30

The melting point diagram (fig. 5) presents an eutectic point at  $T = 446.3 \text{ K}^\circ$  corresponding to a ratio S/R = 72.5 : 27.5. On the basis of this diagram, the definition of racemic compound applies to the equimolecular mixture of the enantiomers present in Sulpiride.

## 2.7 X-Ray Powder Diffraction

The X-ray diffractions of sulpiride and of the (S)-form were determined (5) with a Philips powder diffractometer PW 1710 equipped with nickel-filtered copper radiation  $1.54051 \text{ \AA}$  under the following instrumental conditions:

Tube LFF, Cu/Ni, Al/Ni, 40KV; slits: 1- 0,1-1; scanning speed  $1^\circ \text{ min}^{-1}$ ; paper speed 600 mm/h.

Interplanar distances and relative intensity of the major lines in the X-ray powder diffraction patterns of Sulpiride and (S)-enantiomer are reported in table 5.



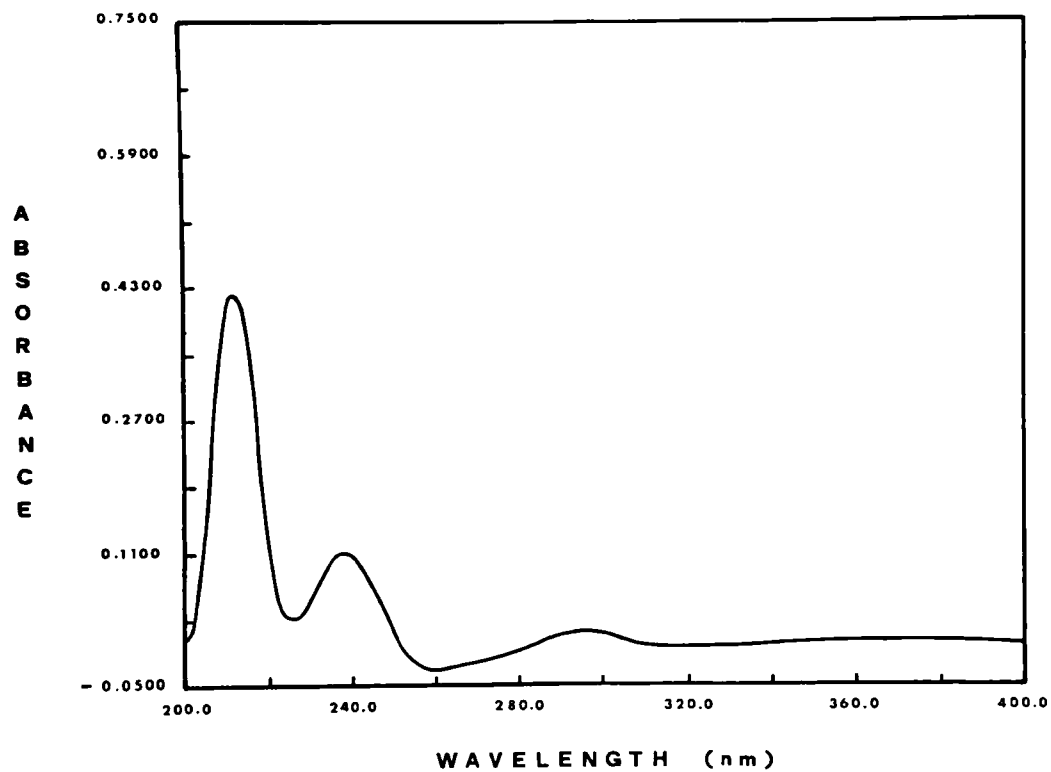


Fig. 6. - Ultraviolet spectrum of sulpiride in methanol

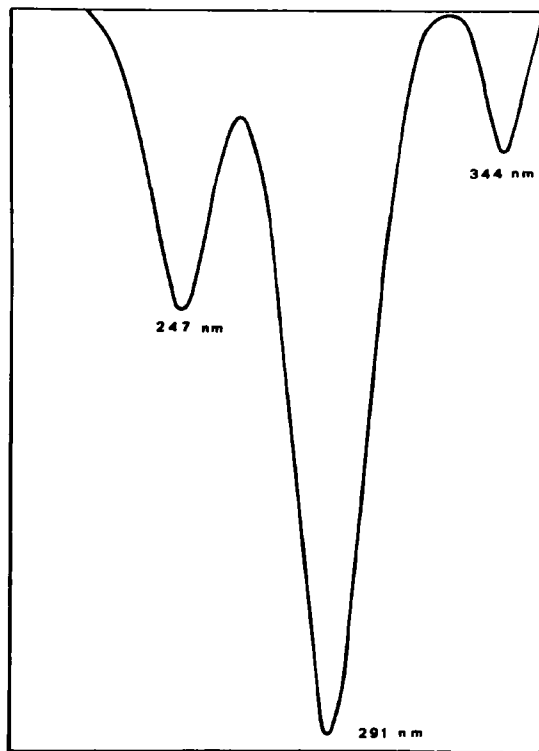


Fig. 7. - Excitation spectra  
of sulpiride in HCl N

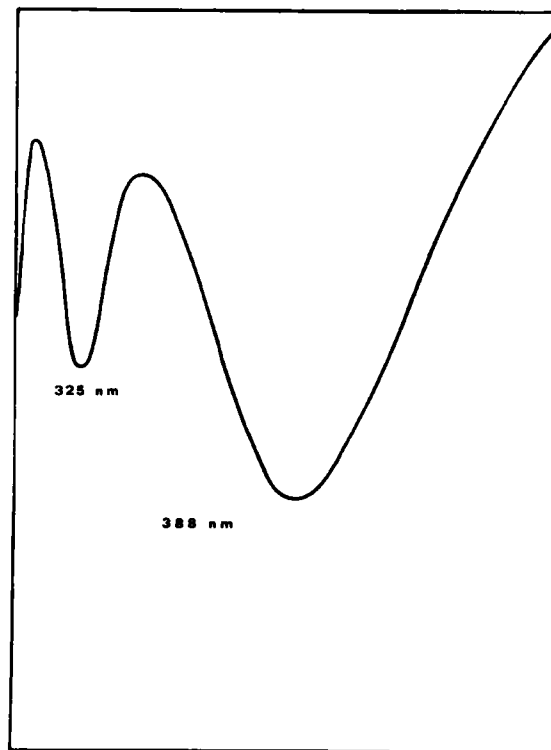


Fig. 8. - Emission spectra  
of sulpiride

Table 5  
X-Ray Powder Diffraction data of Sulpiride and his  
(S)-enantiomer

Sulpiride		(S)-enantiomer	
d(A)	I/I <sub>o</sub>	d(a)	I/I <sub>o</sub>
6.963	41.88	12.117	100.00
6.718	24.26	8.374	17.86
6.367	25.73	5.975	32.63
5.381	29.86	5.215	61.11
5.084	37.16	4.899	84.08
4.599	77.04	4.001	42.82
4.394	68.27	3.796	23.23
4.222	28.61	3.696	39.02
3.819	100.00	3.665	22.06
3.673	16.47	3.620	30.75
3.631	15.54	3.583	19.30
3.101	26.86	3.429	31.06

## 2.8 Crystal Structure

Y.L. Ma et al. (6) have determined the dimension of the unit cells of sulpiride and of its S-enantiomer. The values are reported in table 4. There is a good agreement between these values and those previously reported by C. Houtteman et al. (7) for sulpiride.

Table 4  
Unit cells dimensions of Sulpiride and S-enantiomer

Characteristics	Sulpiride	S-Enantiomer
Crystal structure	Triclinic	Ortorombic
Space Group	P 1	P 2, 2, 2
Cell Dimension	a= 9.067(4) b= 9.372(4) c= 11.175(5)	a= 12.037(6) b= 24.163(4) c= 11.536(6)
Angle	$\alpha$ = 65.62 (9) $\beta$ = 79.83 (9) $\gamma$ = 79.79 (3)	
1 (C)	1.34	1.35
2	2	8
Refined R	7.38%	6.43%
Reflution	1536	1401

The significant dissimilarities between the two X-Ray Powder Diffractions and of the unit cells dimensions allow the conclusion that sulphiride is a true racemic compound.

## 2.9 Melting Range

A careful determination of the melting point of sulphiride gave 178-80° with the Tottoli apparatus and 177.5 with D.S.C.. The values of 185-87° (Tottoli) and 186.7° (D.S.C.) were determined for the (S)-form (4).

## 2.10 Solubility

One part of sulphiride is soluble in about 2200 of water, in 100 of ethanol, in about 220 of chloroform, in about 2600 of ether, and in 50 of methanol (8).

## 2.11 pKa

The ionization constants  $pK_{a1}$  relative to the basic nitrogen of the pyrrolidine ring and  $pK_{a2}$  due to the sulfamoyl group present in sulphiride have been determined (9,10). The pertinent data are collected in table 6.

Table 6  
Ionization Constants of Sulpiride

Methods	Solvents	$pK_{a1}$	$pK_{a2}$	Ref.
Spectrofotom.	H <sub>2</sub> O	8.99	-	9
Potentiometric	H <sub>2</sub> O	9.00	10.19	9
	H <sub>2</sub> O.CH <sub>3</sub> OH 7.7%	8.98	10.05	10
	H <sub>2</sub> O.CH <sub>3</sub> OH 42.4%	8.36	10.48	10

## 2.12 Optical Rotation of Enantiomers

Polarimetric determinations were done on a sample of the S (-) form with an enantiomeric purity of 99.33% (4). The results at different temperatures and concentrations are reported in table 7.

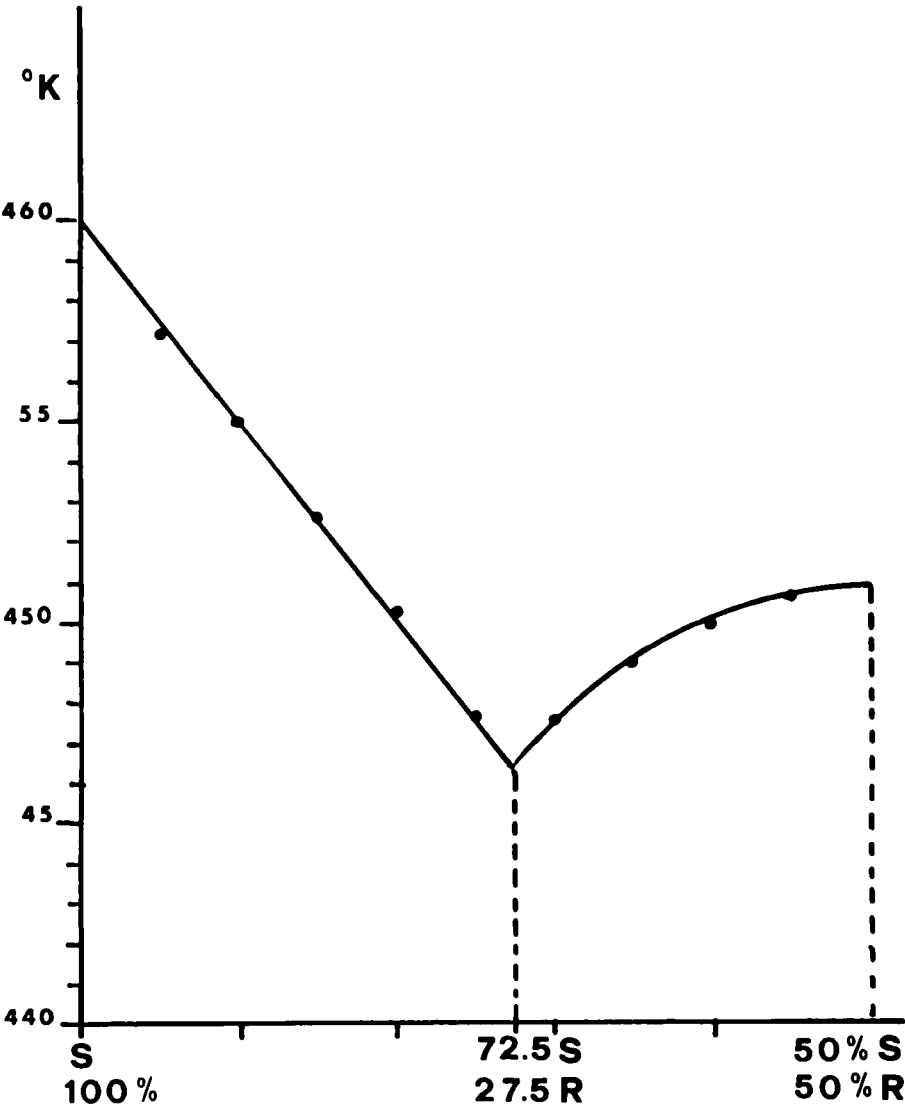


Fig. 9. - Melting point phase diagram of sulpiride

Table 7

C	T°	589	578	546	436	365
0.5	25	-66.6	-70.0	-80.4	-138.6	-223.6
1.5	25	-67.6	-70.1	-80.6	-137.9	-222.3
2.73	15	-67.4	-70.3	-79.91	-138.8	-223.68
2.73	28	-65.8	-68.75	-78.4	-136.3	-219.68

The following measurements are also reported in the literature.

$$[\alpha]_D^{25} = -66.8 \text{ (c = 0.5 DMF) (11)}$$

$$[\alpha]_D^{20} = -67.7 \text{ (c = 2 DMF) (12)}$$

### 2.13 Optical Rotatory Dispersion

The CD spectrum of (-)Sulpiride (Dichrogaph Mark V - Jobin-Yvon) in aqueous solution (0.01N HCl) in the range of the first aromatic band is represented in fig. 10. Sulpiride shows low dichroism ( $\epsilon_{295} = 0.66$ ) when compared to the absorption at the same wave length. (13) At shorter wave lengths a second negative CD band is present, but the very unfavourable ratio signal/noise renders this band less significant.

## 3. MANUFACTURING PROCEDURES

### 3.1 Synthesis of Racemate

Sulpiride is obtained in the easiest way by condensation of a reactive derivative of 2-methoxy-5-sulfamoylbenzoic acid (i.e. chloride, mixed anhydrides, esters, etc.) with 1-ethyl-2-aminomethylpyrrolidine. A convenient preparation of this last compound is the crucial point of the synthesis.

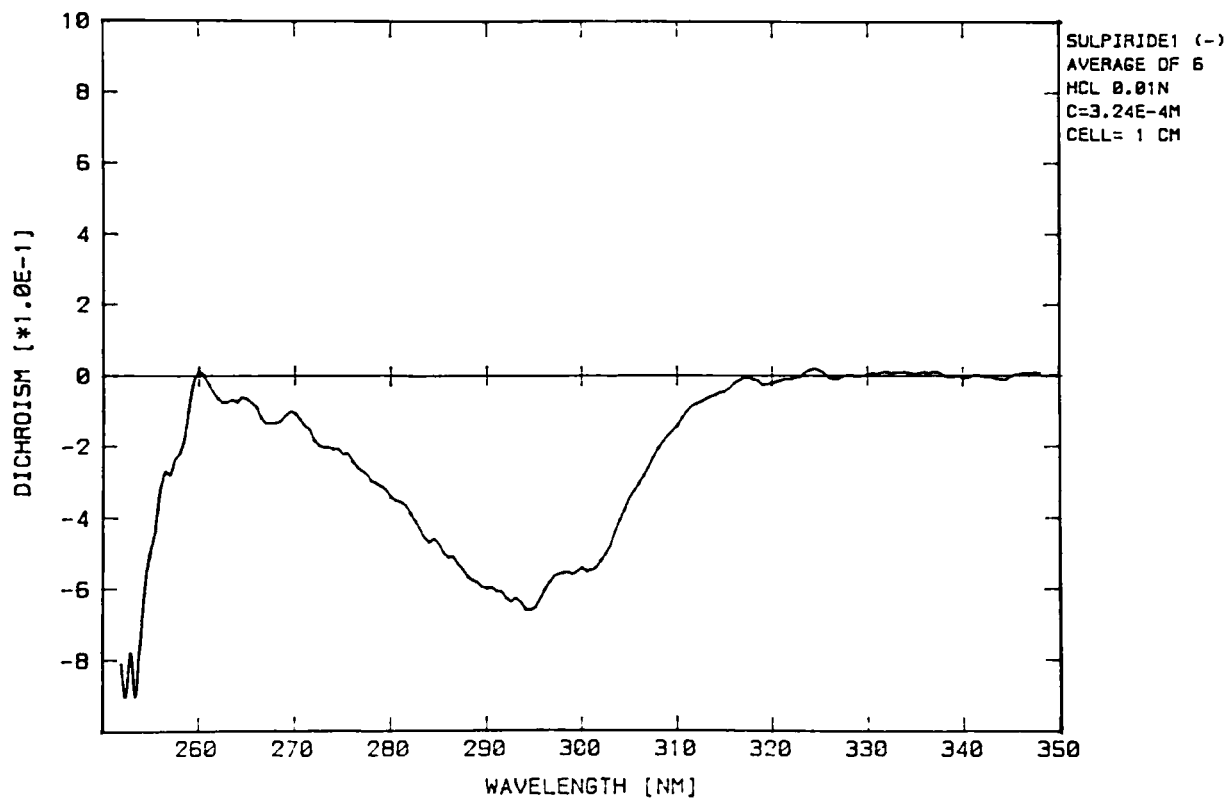
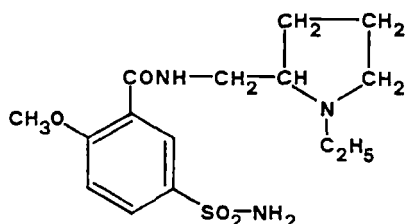
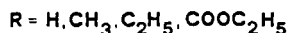
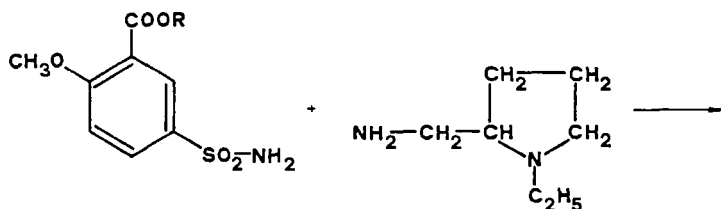


Fig. 10. - CD spectrum of S-form of sulpiride in 0,001N HCl



### 3.1.2 Preparation of $^{14}\text{C}$ -Labelled Sulpiride

The synthesis was studied in order to obtain the labelling at the carbon atom of carboxyl group.

The pathway is presented in the scheme 2. (14,15)

The product obtained has a specific activity of 37.6 mCi/nM (15) and 46.5 mCi/nM (16) with a radiochemical purity of 99%.

A.R. Imondi et al. in their  $^{14}\text{C}$  studies on sulpiride metabolism (17) besides carboxyl- $^{14}\text{C}$ -labelled sulpiride, employed also a sulpiride labelled in the pyrrolidine nucleus (3,4- $^{14}\text{C}$ -pyrrolidine) supplied by New England Nuclear, Boston, Mass.

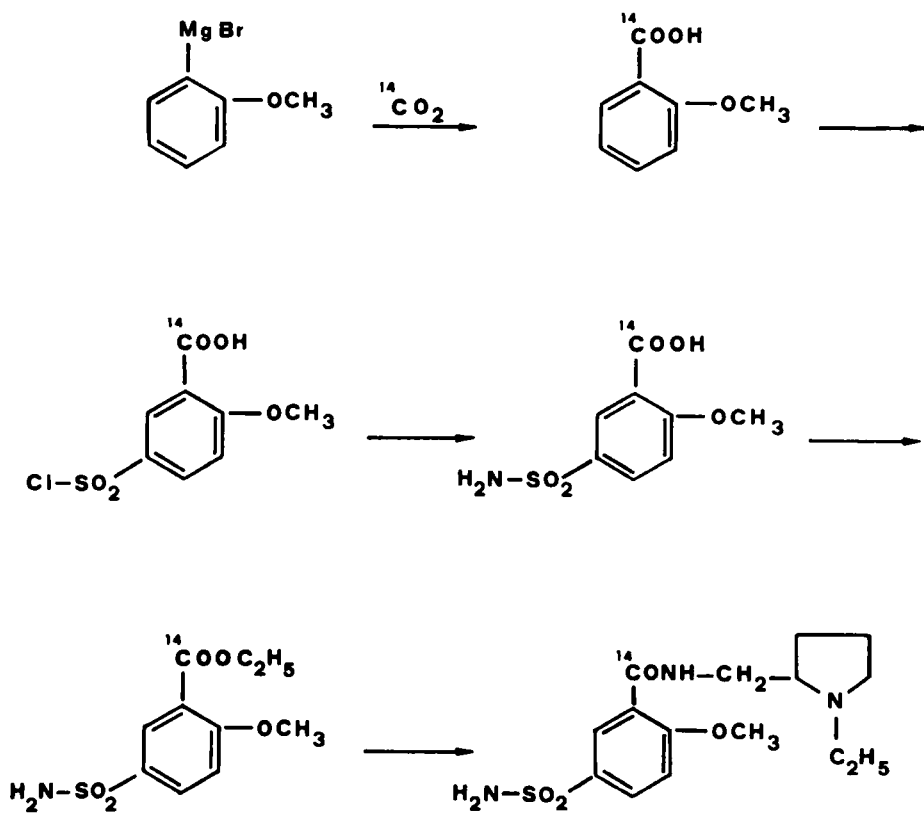
### 3.2 Optical Resolution

The diastereoisomeric salts of sulpiride, obtained with S(-)pyroglutamic acid in 95% ethanol, have been used for the optical resolution. (18)

### 3.3 Absolute Configuration of Enantiomers

(S)-1-ethyl-2-aminomethylpyrrolidine, obtained from (S)-proline was condensed with 2-methoxy-5-sulfamoylbenzoyl chloride. The resulting (S)-5-(aminosulfamoyl) N [(1-ethyl-2-pyrrolidinyl) methyl]-2-methoxybenzamide was levorotatory. (11)



SCHEME 2. SYNTHESIS OF  $^{14}\text{C}$  LABELLED SULPIRIDE

#### 4. STABILITY

Sulpiride as a crystalline solid is very stable, after 60 months of storage no variation was observed in a batch analyzed by HPLC. Similar results have been obtained when sulpiride was maintained for thirty days at 55°; 75°; 100°C. Also daylight and U.V. light did not affect sulpiride even after a month of exposure. (19)

#### 5. METABOLISM AND PHARMACOKINETICS

##### 5.1 Metabolism

When  $^{14}\text{C}$ -sulpiride was administered intraperitoneally to the rat (15,16,20) 64% of radioactivity was detected in the urines and 26% in the feces after 72 hr. Similar experiments after oral administration gave 18% and 74% in urines and feces respectively.

In the average 50-68% of the administered sulpiride in terms of radioactivity can be recovered.

The six metabolites reported in fig. 11 were identified, namely 0.5 hr after oral administration 25% of metabolites were already present in plasma besides 75% of unaltered sulpiride. On the other hand 43.5% of unaltered sulpiride and 37.5% of conjugated metabolites were determined in the urines after 12 hr. The quantities of each metabolite are reported in figure 11.

After oral administration in the dog 99% of radioactivity was present in plasma after 0.5 hr and 85% in the 12 hr urines. In this animal species no metabolites were detected in plasma and the five metabolites in the urines accounted only to 4% of the excreted radioactivity.

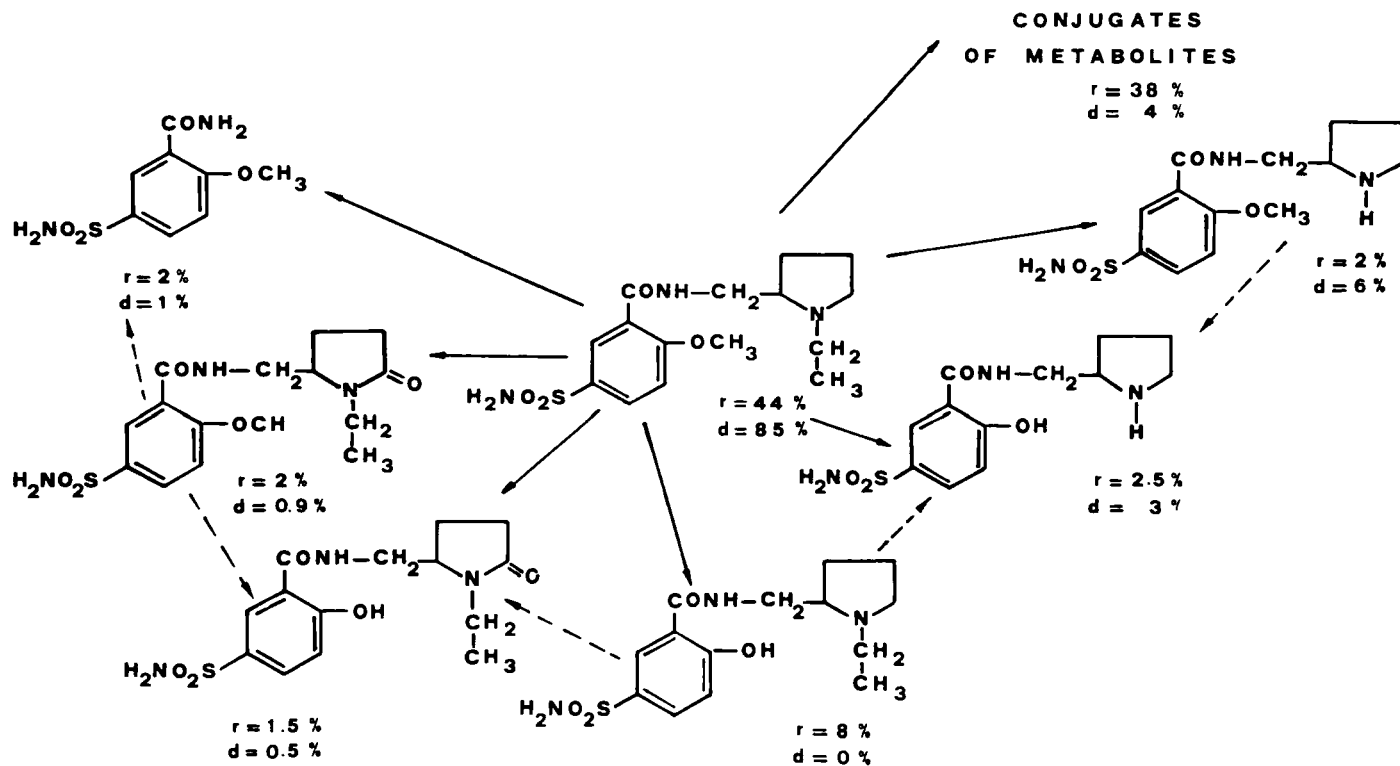
Oral administration was studied also in primates and urines of *Macaca mulatta* contained 10-30% of metabolite V as the major metabolite (21,16).

The metabolic pathway in man is very simple; after oral administration of 100 mg of  $^{14}\text{C}$ -sulpiride to five human volunteers 95% of the drug was recovered unchanged. (16)

##### 5.2 Pharmacokinetics

The pharmacokinetics of sulpiride has been studied in the rat, hamster and dog, following i.v. and oral administration (22,23). In the healthy volunteers (24) after oral administration of 100 mg of sulpiride a slow and incomplete absorption was observed, the recovery of unchanged drug in urine was 15% with a mean renal clearance

Fig 11. METABOLIC PATHWAY IN RAT(r) AND DOG(d)  
Quantities in urine 12 hr after oral administration



of 223 ml/min. After i.v. administration, 70% of the dose was recovered unchanged in urine within 36 h. with a mean renal clearance of 310 ml/min. A mean peak plasma concentration of 0.24 µg/ml was observed 3-4 h after oral administration of 100 mg sulpiride to 10 healthy subjects (25). When 50 mg sulpiride were administered orally to 14 patients (26) a mean value of 0.18 µg/ml was determined after 4-7 h.

The study of intramuscular administration (27) at the dose levels of 50 100 and 200 mg to nine healthy male subjects indicated that sulpiride is excreted unchanged by the renal route.  $93.1 \pm 6.6\%$  of the administered dose was recovered in the urine.

### 5.3 Acute Toxicity

The LD<sub>50</sub> values for the racemic form initially (28) reported in the literature were:

Rats = 2.3 g/kg (p.o.) . 0.18 g/kg (i.p.)

Mice = 9.3 g/kg (p.o.) . 0.23 g/kg (i.p.)

Subsequently the following values for the toxicity in the rat of the two enantiomers were found: (29)

(-) - form: 284.14 (243.36 - 333.77) mg/kg i.p.

(+) - form: 239.74 (204.25 - 281.29) mg/kg i.p.

(+) - form: 204.78 (174.6 - 233.31) mg/kg i.p.

## 6. METHODS OF ANALYSIS

### 6.1 Identification

The following tests are quoted in the Japanese Pharmacopea (Jap. Ph.):

- a) Dissolve 0.01 g of sulpiride in 5 ml of dilute hydrochloric acid and 20 ml of water. To 5 ml of this solution add 5 ml of Dragendorff's reagent: a red-orange precipitate is produced.
- b) To 0.5 g of sulpiride add 3 ml of a solution of sodium hydroxide, and heat: the gas evolved changes moistened red litmus paper to blue.
- c) Dissolve 0.1 g of sulpiride in 0.1N sulfuric acid to make 100 ml. To 5 ml of this solution add water to make 50 ml and determine the absorption spectrum of this solution using water as the blank: it exhibits a maximum between 289 nm and 293 nm.

## 6.2 Non-Aqueous titration

The Jap. Ph. describes the following method:

Weigh accurately about 0.4 g of sulpiride, previously dried, dissolve it in 80 ml of glacial acetic acid, and titrate with 0.1N perchloric acid until the color of the solution changes from purple through blue to blue-green (indicator: 2 drops of methylrosaniline chloride TS).

Perform a blank determination in the same manner and make any necessary correction.

Each ml of 0.1N perchloric acid = 34.143 mg  $C_{15}H_{23}N_3O_4S$ .

## 6.3 Colorimetric Analysis

Sulpiride forms coloured complexes which can be extracted in organic solvents, so that a colorimetric determination is possible. Bromothymol blue at pH 8 gives rise to a coloured substance extractable in chloroform; the absorbance is determined at 620 nm. With this method concentration of 0.5 mcg/ml of sulpiride can be determined in serum. (30)

## 6.4 Ultraviolet Spectrophotometric Analysis

Direct determination of sulpiride can be done spectrophotometrically at 291 nm ( $E_{1\text{cm}}^{1\%} = 71.4$ ) after extraction with chloroform of an aqueous solution at pH 10. Concentration in the range of 1 mcg/ml of plasma can be determined. (30)

## 6.5 Fluorometric Analysis

Samples of serum and urine were adjusted with 0.1 N NaOH to pH 10 and extracted with chloroform. The chloroform liquors were in turn extracted with 2N HCl. The acidic layers were centrifuged and excited at 280 nm. The emission maximum at 342 nm was determined.

Linear results were observed for concentration in the range of 1-5 mcg/ml with a recovery of about 80%. (31)

A fluorescence detector with excitation maximum at 299 nm and emission maximum at 342 nm has been used for the quantitation of sulpiride in HPLC. (32)

## 6.6 Radio-Immunoassay

Using  $^3\text{H}$ -sulpiride as a tracer an antiserum was obtained by immunization of rabbits with sulpiride albumin conjugate. A standard curve relating the concentration of sulpiride between 0.1 and 500 ng was established (33).

Two patent applications were filed (34,35) concerning the preparation of the enzyme immunoassay.

The radioimmunoassay has been used for the determination of sulpiride in different areas of rat brain (hypothalamus, medulla oblongata, cerebellum, striatum, mesolimbic area and hippocampus). (36)

### 6.7 Paper Chromatography

Descending chromatography was performed on S+S 2043 B Mgl paper (Schleicher and Schull) and developed in n-butanol:water: 25% ammonia sol-80:10:10.

Detection with Dragendorff and ninhydrin reagents. (16)

### 6.8 Thin-Layer Chromatography

The methods for separation and detection of sulpiride are summarized in table 8.

Table 8

Solvent system	Plate	R <sub>f</sub> x100	Reference
I	A	-	(30)
II	B	-	(37)
III	C	55	(16)
IV	C	54	(16)
V	C	13	(16)
VI	C	15	(16)
VII	C	14	(16)
VIII	C	10	(20)
IX	C	50	(20)
X	C	74	(20)
XI	C	18	(15)

#### Solvent system:

- I n-butanol:acetic acid:water - 100:80:5
- II acetone:n-butanol:water:aqueous ammonia - 66:30:3:1
- III chloroform:methanol:25% ammonia sol.- 60:40:2
- IV isopropanol:toluene:25% ammonia sol.- 60:30:10
- V benzene:methanol:acetic acid - 60:35:5
- VI n-butanol:methanol:water:acetic acid - 60:25:25:5
- VII methanol
- VIII ethyl acetate:methanol:formic acid:acetonitrile - 60:15:10:15

- IX acetoneitrile:methanol:formic acid - 50:15:6  
X ethyl acetate:methanol:25% aq.ammonia:acetonitrile  
60:15:20.5:1.5  
XI butanol:acetic acid:water - 60:20:20

Detection:

Ultraviolet light (293 nm)  
Dragendorff reagent  
Ninhydrin reagent

Plate

- A Polyamide Kodak 511 V  
B Glassplate 20x20 cm coated with silica gel  
C Kieselgel 60 F<sub>254</sub>

6.9 Gas Liquid Chromatography

Determination of sulpiride in biological fluids has been reported by A. Frigerio and C. Pantarotto (38). The sample was extracted from a basic solution with ethyl acetate, the solvent evaporated and trimethylbenzylammonium hydroxide and chlorthalidone (as a standard in a methanolic solution) were added to the residue. The trimethyl derivative of sulpiride was then injected in the gas-chromatograph equipped with a flame-ionization detector. The following operating conditions were observed: glass column (2m x 4mm I.D.) packed with 3% OV-17 on Gas-Chrom Q, 100-120 mesh conditioned for 1h at 250° with a N<sub>2</sub> flow-rate of 30 ml/min, 4h at 310° with no N<sub>2</sub> and 24h at 280° with a N<sub>2</sub> flow of 35 ml/min. Injection temperature 300°. This method was applied also to chemical ionization-mass fragmentography measurements. (CI-MF).

6.10 High Performance Liquid Chromatography

N. Verbieese-Genard et Al. (39) have chosen the following conditions for reverse phase high pressure liquid chromatography:

- stationary phase: octadecyl Sil X 1 particle size 10 µm (column 0.25 m x 26 mm).
- mobile phase: acetonitrile 10% v/v, phosphate buffer 0.3M 90% v/v, pH = 6.0; flow rate 2.0 ml/min at T=40°. U.V. detector at 215 nm.

This method is suitable for the determination of sulpiride in pharmaceutical dosage-forms such as syrups and tablets. Another reverse-phase system applied to a very broad selection of drugs including sulpiride was reported by D.L. Massart et Al. (40)

Operating conditions were:

- stationary phase: Lichrosorb CN (10  $\mu$ m) in stainless steel columns 250 x 4 mm I.D.
- mobile phase: acetonitrile-water-propylamine (49:51:0.01), flow rate 1 ml/min at room temperature
- U.V. detector at 254 nm.

A quantitative determination of sulpiride in biological fluids was described also by G. Alfredsson et Al. (32). They also used a reverse-phase system with a fluorescence detector (excitation at 299 nm and emission at 342 nm). F. Bressolle and Bres (41) described an HPLC method with detection limit of 10 ng/ml in plasma and of 15 ng/ml in the urine.

- stationary phase: Lichrosorb RP8 Merck (10  $\mu$ m) column 250 x 4.6 mm I.D.
- mobile phase: 0.1 M ammonium acetate - methanol (10:90) flow rate 1 ml/min T=50°. Pressure 43 bars.
- U.V. detector at 226 nm.

## 7. DETERMINATION IN BODY FLUIDS AND TISSUES

Different amounts of sulpiride were recovered from body fluids and tissues depending on the conditions and solvents used by the different Authors. Whereas extraction with ethyl acetate from solution made basic with 0.1N NaOH gave recoveries in the range of 52-82% (38) use of solutions buffered to pH = 10 and extraction with chloroform allowed recoveries of 98% (27,41).

A summary of different methods of determination in the various biological fluids and tissues is given below:

### Blood (Plasma Serum)

- Colorimetry: (30)
- Spectrophotometry: (30)
- Spectrofluorimetry: (31)
- TLC: (30,37)
- HPLC: (32)(41)(27)(43)(44)(45)
- GLC-Mass spectrometry: (38)
- HPLC-Mass fragmentography (48)



Urine

- Colorimetry: (30)
- Spectrophotometry: (30)
- Spectrofluorimetry: (21)
- TLC: (37)
- HPLC: (42)(32)(27)(41)
- Radioactivity with  $^{14}\text{C}$  sulpiride: (16)(17)(23)
- GLC-Mass spectrometry: (38)

Liver

- GLC-Mass spectrometry: (38)

Milk

- Fluorometry: (46)

Brain

- GLC-Mass spectrometry: (38)

Cerebrospinal fluid

- HPLC: (32)
- HPLC and Mass fragment: (47)

Feces

- Radioactivity with  $^{14}\text{C}$  sulpiride: (16)(17)(23)

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VERAPAMIL

Zui L. Chang

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## 1. Description

### 1.1 Nomenclature

#### 1.11 Chemical Name

- (a) 5-[(3,4-Dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile Hydrochloride
- (b)  $\alpha$ -[3-[[2-(3,4-Dimethoxyphenyl)ethyl]-methylamino] propyl]-3,4-dimethoxy-(1-methylethyl)benzene-acetonitrile Hydrochloride

#### 1.12 Generic Name

Verapamil

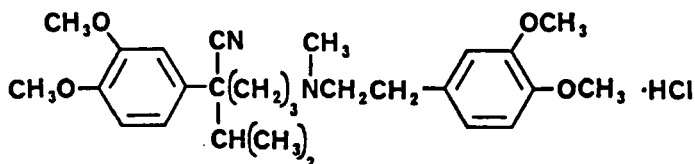
Verapamil Hydrochloride

#### 1.13 Trade Name

Calan™

Isoptin®

### 1.2 Formulas and Molecular Weight



C<sub>27</sub>H<sub>38</sub>N<sub>2</sub>O<sub>4</sub>

M.W. 491.07

### 1.3 Appearance, Color, Odor

Verapamil Hydrochloride is a white crystalline powder with no discernible odor.

## 2. Physical Properties

### 2.1 Infrared Spectrum

The infrared spectrum of verapamil hydrochloride is presented in Figure 1. The spectrum was measured in the solid state as a potassium bromide dispersion. The following bands ( $\text{cm}^{-1}$ ) have been assigned for Figure 1 (1).

a. Between  $3030$  and  $2860 \text{ cm}^{-1}$

A broad complex absorption due to superimposing C-H stretching vibrations of the methyl and methylene groups.

b.  $2840 \text{ cm}^{-1}$

A band due to C-H stretching vibrations of the methoxy groups.

c. Between  $2800$  and  $2300 \text{ cm}^{-1}$

A broad complex absorption due to N-H stretching vibrations of the protonated amine.

d.  $2236 \text{ cm}^{-1}$

A sharp weak band due to C=N stretching vibrations of the saturated alkyl nitrile.

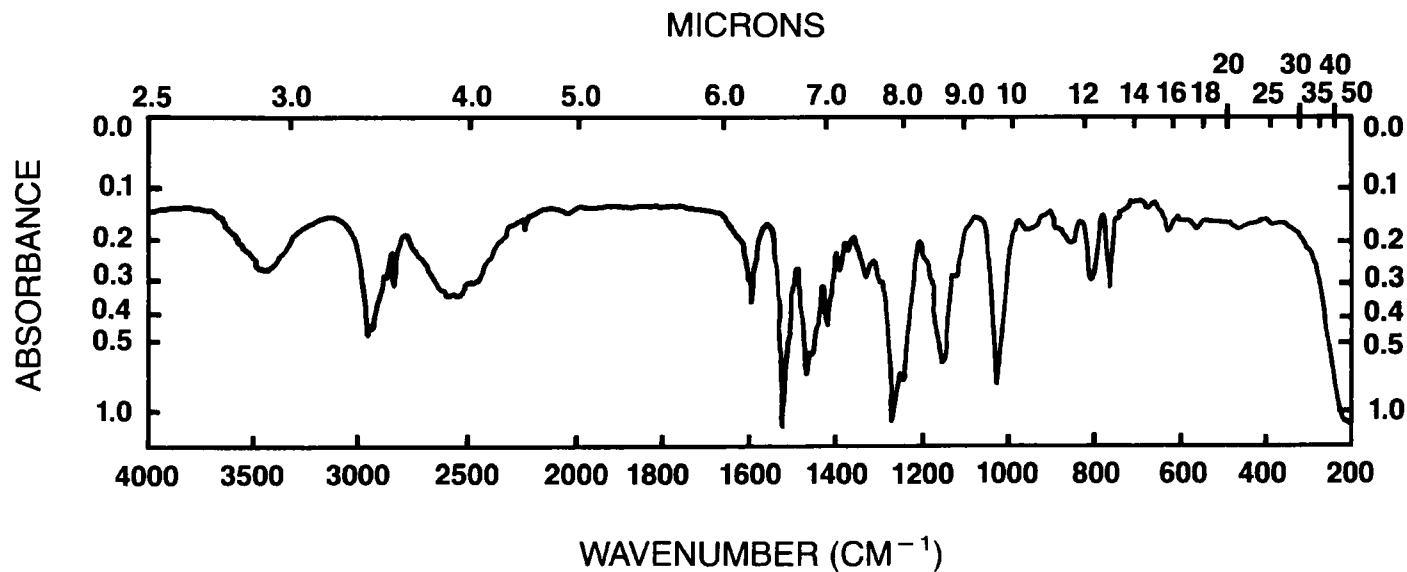
e.  $1607$ ,  $1591$  and  $1518 \text{ cm}^{-1}$

Bands due to skeletal stretching vibrations of the benzene ring.

f.  $1262 \text{ cm}^{-1}$

A strong band due to C-O stretching vibrations of the aromatic ethers.

**Figure 1 – Infrared Spectrum of Verapamil Hydrochloride**



## 2.2 Raman Spectrum

The Raman spectrum of verapamil hydrochloride as shown in Figure 2 was obtained in the solid state on a Cary Model 83 Spectrophotometer. The following bands ( $\text{cm}^{-1}$ ) have been assigned for Figure 2 (2).

a. Between  $3020$  and  $2860 \text{ cm}^{-1}$

A complex of weak bands due to superimposing C-H stretching vibrations of the various methyl and methylene groups.

b.  $2840 \text{ cm}^{-1}$

A band due to C-H stretching vibrations of the methoxyl groups.

c.  $2236 \text{ cm}^{-1}$

A sharp band due to  $\text{C}\equiv\text{N}$  stretching vibrations of the saturated alkyl nitrile.

d.  $1611 \text{ cm}^{-1}$

A band due to skeletal stretching vibrations of the benzene rings.

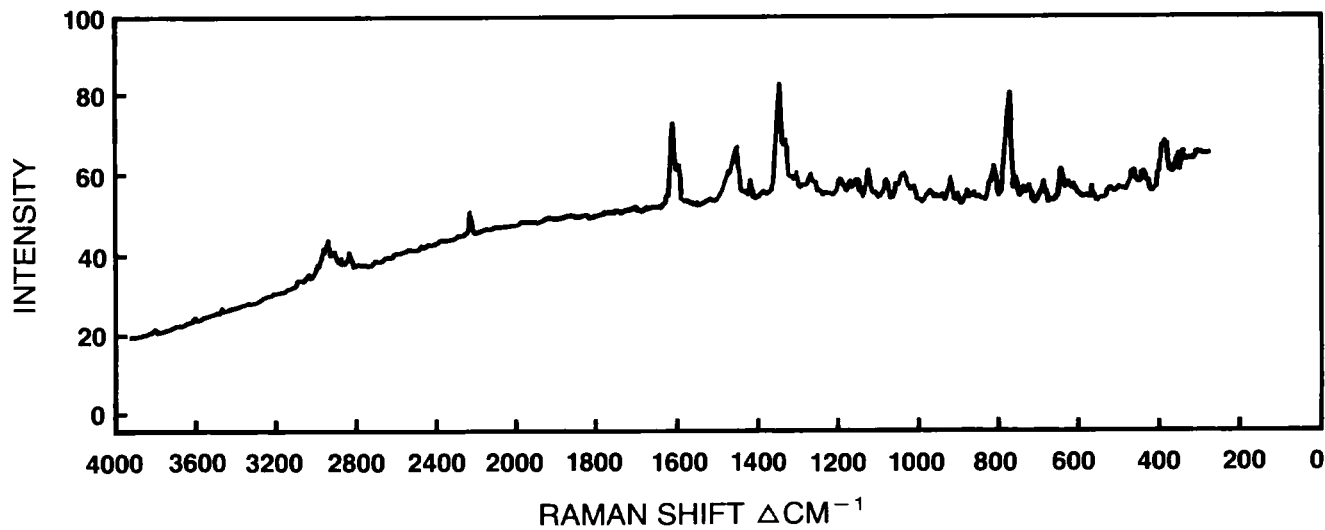
e.  $775 \text{ cm}^{-1}$

A characteristic of 1,2,4-trisubstituted benzenes.

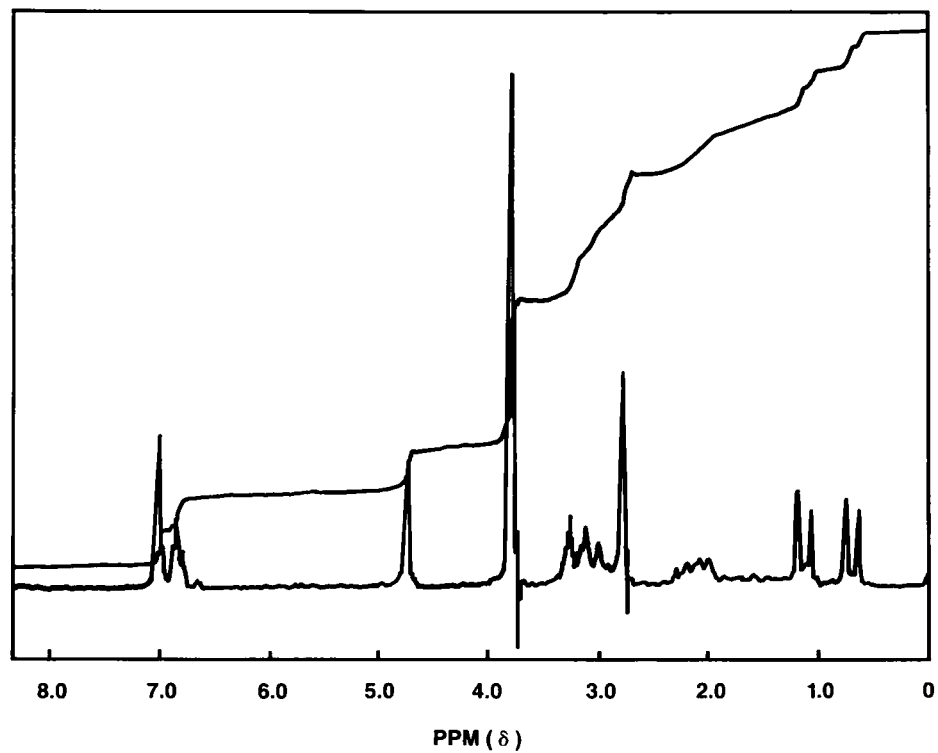
## 2.3 Proton Nuclear Magnetic Resonance Spectrum (PMR)

The proton magnetic resonance spectrum of verapamil hydrochloride as shown in Figure 3 was obtained on a Varian Associates XL100-15 PMR Spectrometer with a Nicolet Technology Corp. TT-100 Computer as a 10% w/v solution in a solvent of deuterated methanol. The spectral peak assignments (3) are presented in Table I.

**Figure 2 – Raman Spectrum of Verapamil Hydrochloride**



**Figure 3 – Proton Nuclear Magnetic Resonance Spectrum of Verapamil Hydrochloride**



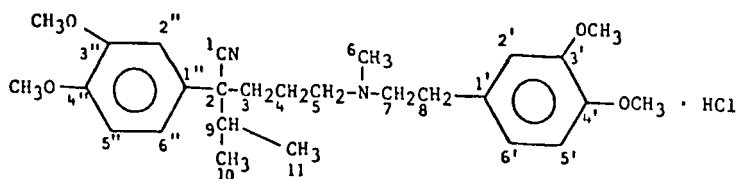
Structure

Table I

Spectral Assignments for  
PMR Verapamil Hydrochloride

<u>Proton Assignment</u>	<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>
$\left\{ \begin{array}{l} 2' - \text{H} \\ 5' - \text{H} \\ 6' - \text{H} \\ 2'' - \text{H} \\ 5'' - \text{H} \\ 6'' - \text{H} \end{array} \right.$	6.66-7.15	Multiplet
$\left\{ \begin{array}{l} \text{OCH}_3 \\ \text{OCH}_3 \end{array} \right.$	3.83	Singlet
$\text{OCH}_3$	3.86	Singlet
$\text{OCH}_3$	3.81	Singlet
$\left\{ \begin{array}{l} 5 - \text{CH}_2 \\ 7 - \text{CH}_2 \\ 8 - \text{CH}_2 \end{array} \right.$	2.75-3.40	Multiplet
$\text{N-CH}_3$	2.82	Singlet
$\left\{ \begin{array}{l} 9 - \text{CH} \\ 3 - \text{CH}_2 \\ 4 - \text{CH}_2 \end{array} \right.$	1.26-2.45	Broad Multiplet
$\left\{ \begin{array}{l} 10 - \text{CH}_3 \\ 11 - \text{CH}_3 \end{array} \right.$	1.22 0.7	Doublet Doublet

All chemical shifts are measured from internal TMS (0.0 ppm).

The peak at 3.32 ppm is due to the solvent used ( $\text{CD}_3\text{OD}$ ), and the peak at 4.82 ppm is due to  $\text{H}_2\text{O}$ .

#### 2.4 Carbon-13 Nuclear Magnetic Resonance Spectrum (CMR)

The carbon-13 nuclear magnetic resonance spectrum of verapamil hydrochloride as shown in Figure 4 was obtained on a JEOL FX902 CMR Spectrometer with a 20% w/v solution in a solvent of deuterated methanol. The spectral peak assignments (4) are present in Table II.



**Figure 4 – Carbon-13 Nuclear Magnetic Resonance Spectrum of  
Verapamil Hydrochloride**

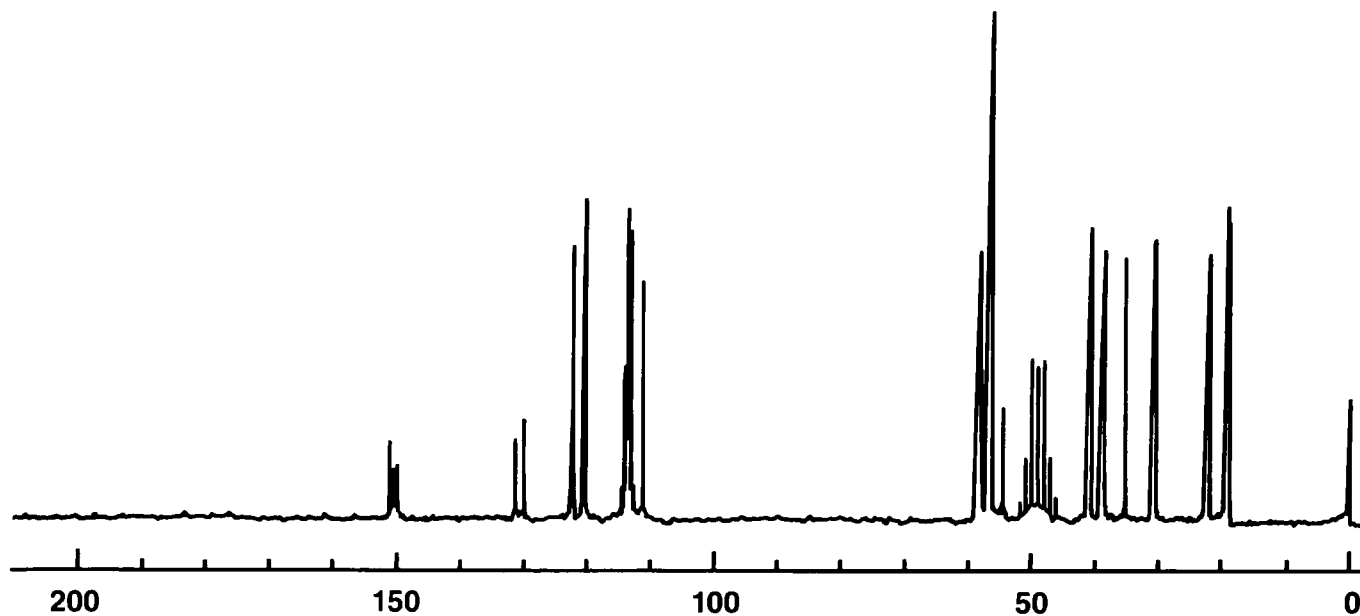


Table II  
 CMR Spectral Assignments  
 for Verapamil Hydrochloride

Carbon Assignment	Chemical Shift, ppm	Multiplicity, ORSFD*
{ C-3'	151.0	Singlet
{ C-3"	150.9	Singlet
{ C-4'	150.4	Singlet
{ C-4"	149.8	Singlet
{ C-1'	131.3	Singlet
{ C-1"	130.1	Singlet
C-1	122.2	Singlet
{ C-6'	122.2	Doublet
{ C-6"	120.5	Doublet
{ C-5'	113.9	Doublet
{ C-5"	113.6	Doublet
{ C-2'	113.1	Doublet
{ C-2"	111.3	Doublet
{ C-7	58.1	Triplet
{ C-5	56.9	Triplet
OCH <sub>3</sub> x 4	56.6	Quartet
C-2	54.6	Singlet
C-6	40.7	Quartet
C-9	38.6	Doublet
{ C-8	35.4	Triplet
{ C-3	30.6	Triplet
{ C-4	22.0	Triplet
{ C-10	19.3	Quartet
{ C-11	19.0	Quartet

\*Off resonance single frequency decoupling experiment.

All chemical shifts were measured from internal TMS. The multiplet at 49.0 ppm is due to the solvent  $\text{CD}_3\text{OD}$ .

## 2.5 Mass Spectrum

The mass spectrum of verapamil as shown in Figure 5 was obtained using a Kratos Model MS-50 Mass Spectrometer with an ionization electron beam energy of 70 eV. High resolution data were compiled and tabulated with the aid of Kratos DS-55 Software.

The mass spectrum assignments of the prominent ions and subsequent fragments are shown in Table III and Figure 6(5).

Table III

High Resolution Mass of Verapamil Hydrochloride

<u>Measured Mass</u> (m/e)	<u>Calculated Mass</u>	<u>Formula</u>
453.2767	453.2753	$\text{C}_{27}\text{H}_{37}\text{N}_2\text{O}_4$
303.2080	303.2073	$\text{C}_{18}\text{H}_{27}\text{N}_2\text{O}_2$
151.0759	151.0759	$\text{C}_9\text{H}_{11}\text{O}_2$

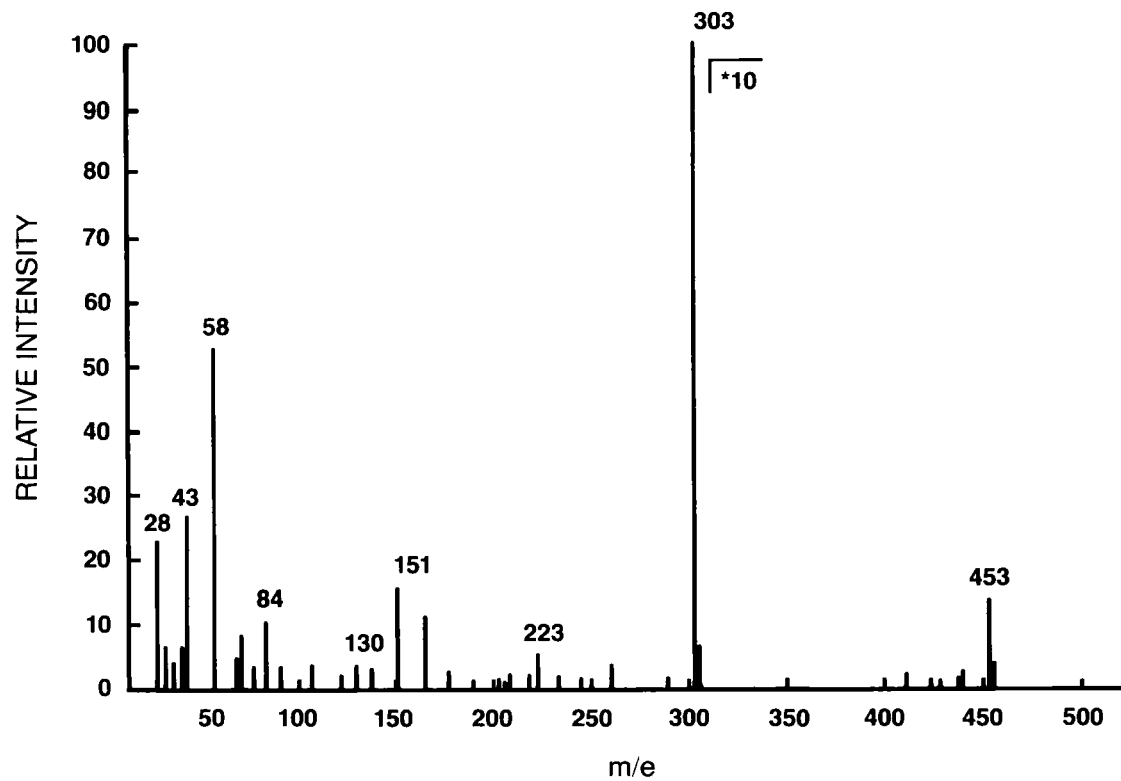
## 2.6 Ultraviolet Spectrum (UV)

When the UV spectrum of 0.002% solution of verapamil hydrochloride in methanol was scanned from 350 to 200 nm, two maxima at 230 nm ( $\epsilon = 16,700$ ) and 278 nm ( $\epsilon = 6,090$ ) were observed (Figure 7).

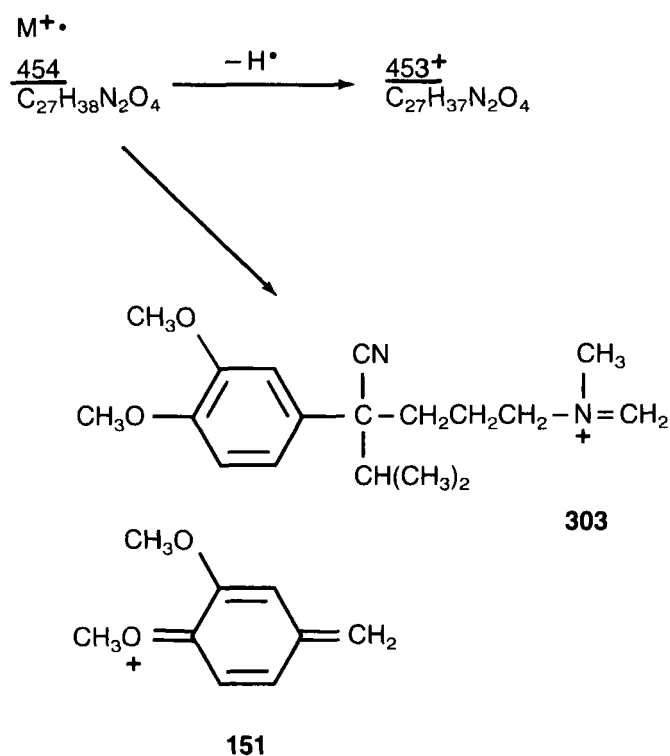
## 2.7 Fluorescence Spectrum

A 0.005% solution of verapamil hydrochloride in methanol exhibits fluorescence when excited with ultraviolet light. The emission spectra of verapamil hydrochloride in methanol, given in Figure 8 show an emission maximum at 316 nm (6). These spectra were obtained using

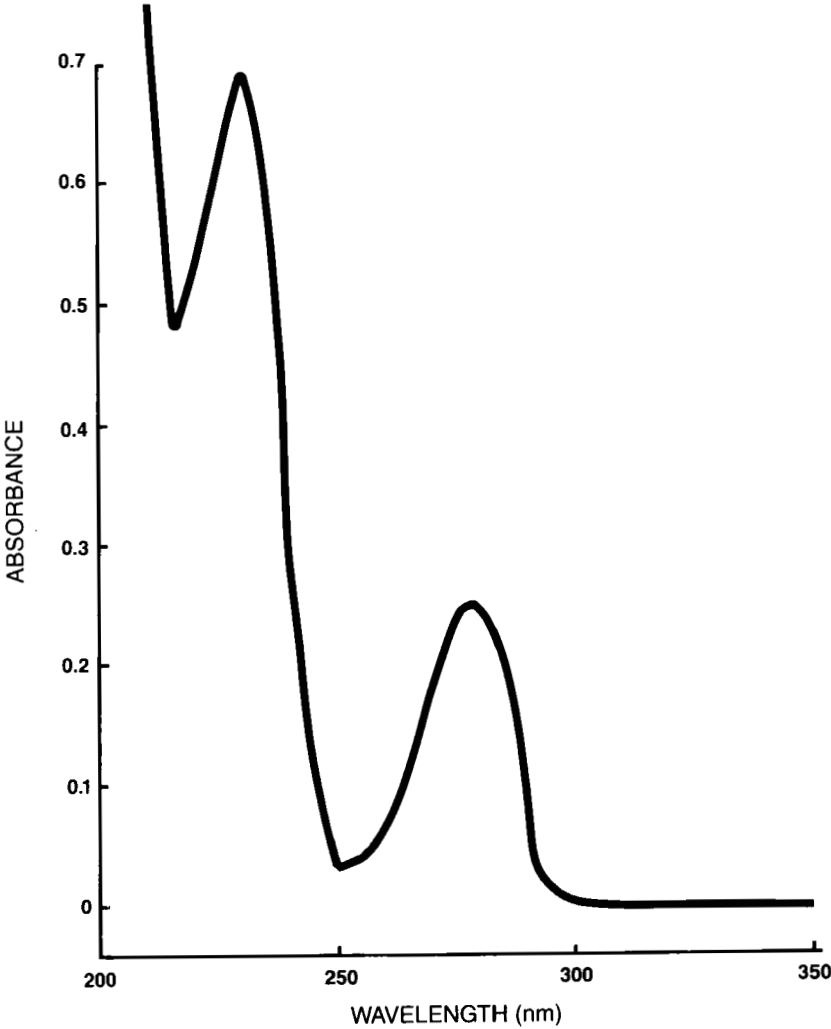
**Figure 5 – Mass Spectrum of Verapamil Hydrochloride**



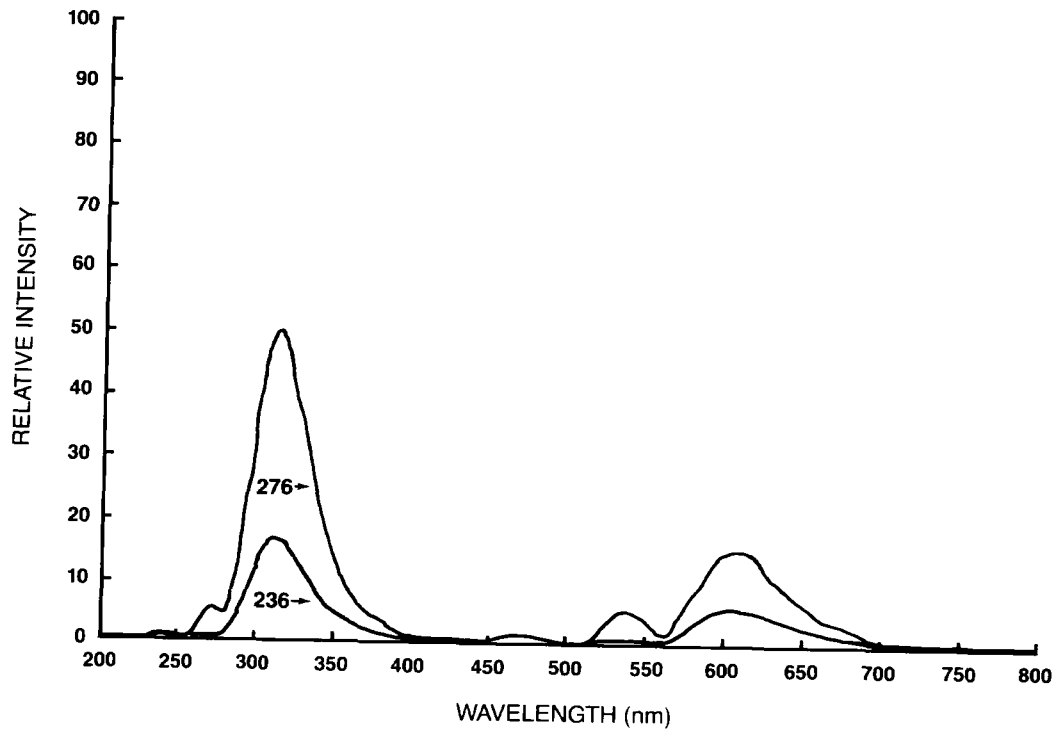
**Figure 6 – Fragmentation  
Pathway of Verapamil Hydrochloride**



**Figure 7 – Ultraviolet Absorption Spectrum of Verapamil Hydrochloride**



**Figure 8 – Emission Spectra of Verapamil Hydrochloride  
Excitation Wavelength at 236 nm and 276 nm**



excitation wavelengths of 236 nm and 276 nm which correspond to the maxima of the absorption spectra.

## 2.8 Solubility

The solubility of verapamil hydrochloride as a function of pH is shown in Table IV. Solubility is 80-90 mg/mL from pH 2.3 to 6.4 where the ionized species predominates, then decreases rapidly at high pH. The solubility of the base form of verapamil hydrochloride is 0.025 mg/mL in 0.1 N NaOH (7).

Table IV

Solubility of Verapamil Hydrochloride in  
Various pH Solutions at 25°C

<u>Solvent</u>	<u>Solubility, mg/mL</u>
Water, adjusted to pH 2.32	82
Water, adjusted to pH 3.05	78
Water, adjusted to pH 4.65	89
Water, adjusted to pH 4.86	82
Water, adjusted to pH 5.59	76
Water, adjusted to pH 6.35	83
Water, adjusted to pH 6.54	46
Water, adjusted to pH 6.59	29
Water, adjusted to pH 6.76	11
Water, adjusted to pH 7.32	0.44
Water, adjusted to pH 8.09	0.17
Water, adjusted to pH 8.87	0.062
0.1 <u>N</u> NaOH (pH 12.6)	0.025

A 0.1 N sodium hydroxide solution and a 0.1 N hydrochloric acid solution were used for adjustment of pH of various solutions.

The following solubility data have been determined for verapamil hydrochloride at room temperature:



<u>Solvent</u>	<u>Solubility, mg/mL</u>
1. Water	83
2. Ethanol, 200 proof	26
3. Propylene Glycol	93
4. Ethanol, 190 proof	>100
5. Methanol	>100
6. 2-Propanol	4.6
7. Ethyl Acetate	1.0
8. Dimethylformamide	>100
9. Methylene Chloride	>100
10. Hexane	0.001

## 2.9 X-Ray Powder Diffraction

The x-ray powder diffraction pattern of verapamil hydrochloride was determined by visual observation of a film obtained with a 143.2 mm Debye-Scherrer Powder Camera (Table V). An Enraf-Nonius Diffractis 601 Generator; 38KV and 18 ma with nickel filtered copper radiation,  $\lambda = 1.5418$ , was employed (8).

Table V

## X-Ray Powder Diffraction Pattern of Verapamil Hydrochloride, d-Spacings and Intensities

$d(\text{\AA})$	$I/I_0^{**}$	$d(\text{\AA})$	$I/I_0^{**}$
18.5	5	3.84	20
10.3	7	3.73	45
8.3	5	3.51	20
7.7	5	3.45	15
6.9	10	3.35B	35
6.7	10	3.19	3
6.3	3	3.12	5
6.1	100	2.87	1
5.9	1	2.79B	3
5.25	30	2.6B	2
5.15	5	2.34	1
4.9	40	2.19B	2
4.7	30	2.15	1
4.6	1	2.05	1
4.35	50	1.91	1
4.15	3		

B = Broad line.

$$*d = \frac{n\lambda}{2 \sin \theta} = \text{interplanar distance}$$

\*\* $I/I_0$  = Relative intensity (based on the highest intensity of 100).

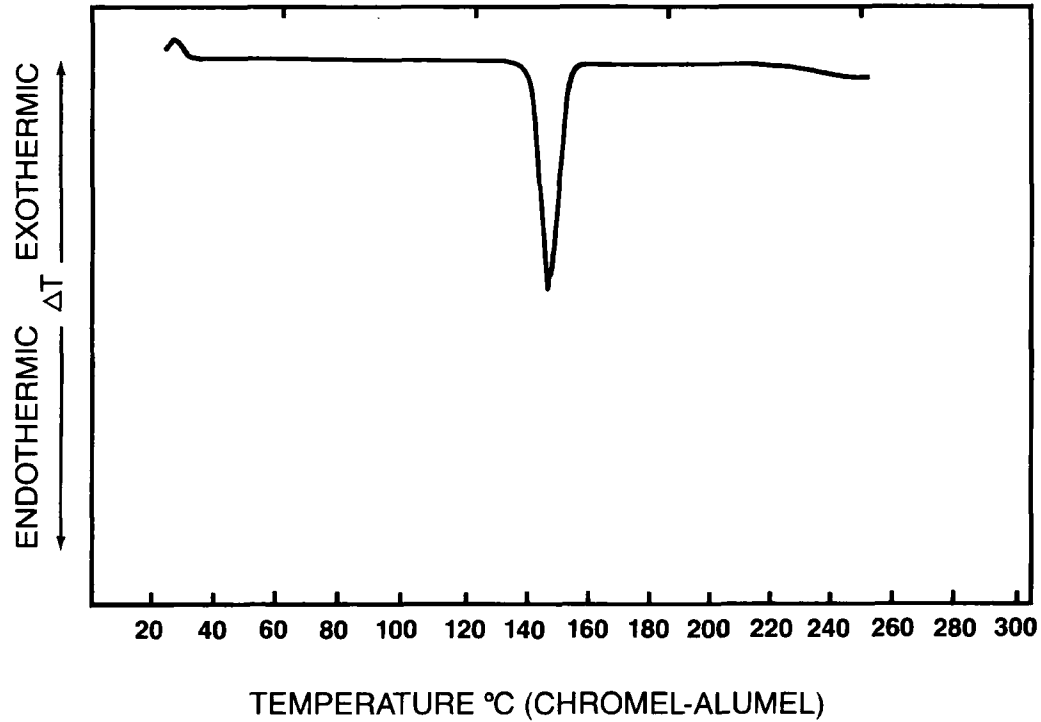
## 2.10 Melting Range

Verapamil hydrochloride melts in the range of 140° and 144°C (Class Ia, USP XXI, p.1250)

## 2.11 Differential Scanning Calorimetry

The differential scanning calorimetry curve of verapamil hydrochloride obtained (9) on a DuPont Model 910 Differential Scanning Calorimeter and Model 900 Thermal Analyzer at a scan rate of 10°C/minute exhibits a sharp melting endotherm with an onset temperature of 143°C and a peak temperature of 148°C as shown in Figure 9. There was no evidence of decomposition up to 250°C.

**Figure 9 – Differential Scanning Calorimeter Analysis Curve of Verapamil Hydrochloride**



### 2.12 pKa

Titration of verapamil hydrochloride with 0.1 N KOH in methanol using methanol-water as the sample solvent and extrapolation to pure water gave a  $pK_a$  value of 8.6 (proton lost).

### 2.13 Hygroscopicity

Verapamil hydrochloride showed negligible hygroscopicity. A sample of verapamil hydrochloride was subjected to 79% relative humidity at room temperature overnight, and only 0.47% moisture absorption was observed (7).

### 2.14 Optical Rotation

A 1% methanol solution of verapamil hydrochloride exhibited no optical activity when measured on a Perkin-Elmer Model 241 Polarimeter at 589 nm in a 1 dm cell at 25°C.

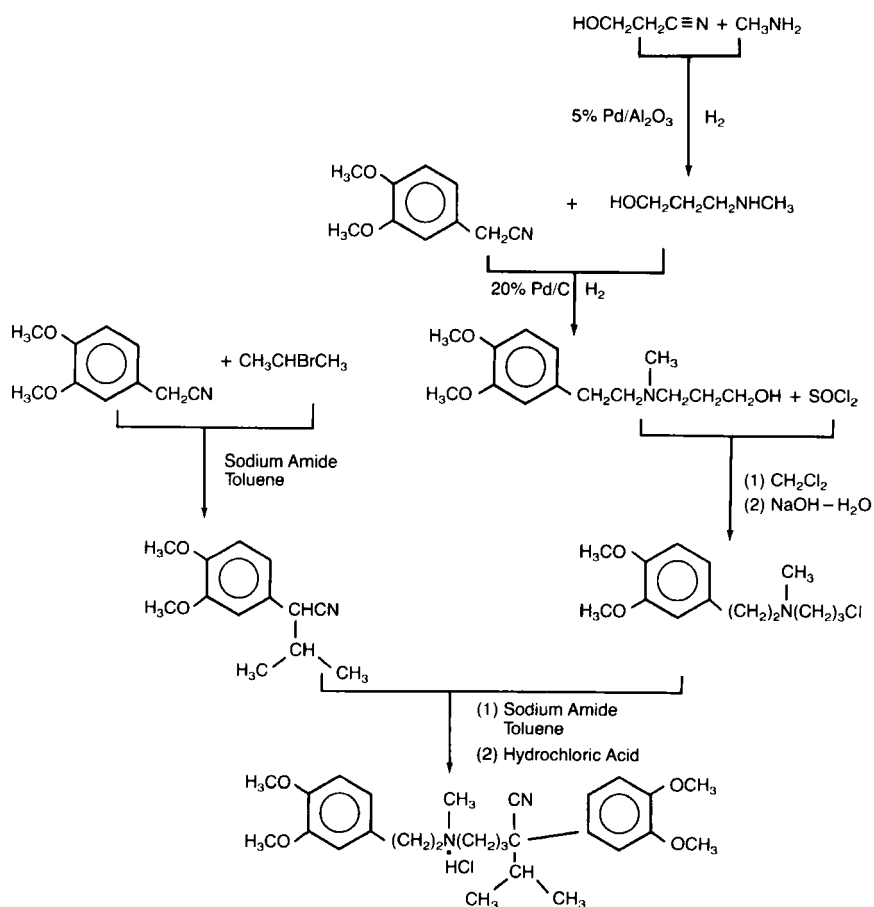
## 3. Synthesis

A Japanese patent was issued to Teikoku Hormone Manufacturing Co., Ltd. in 1978 for the synthesis of verapamil (10). Verapamil also may be synthesized by the reaction scheme shown in Figure 10 (11).

## 4. Stability

In the solid state, verapamil hydrochloride is very stable under high-stress thermal and photochemical degradative conditions. It is also very stable under neutral, acidic and basic aqueous reflux conditions. However, the compound when dissolved in methanol and subjected to UV light for 2 hours showed rapid degradation (52%).

Figure 10 – Synthetic Pathway of Verapamil Hydrochloride



## 5. Method of Analysis

### 5.1 Identification

Verapamil hydrochloride may be identified by infrared spectrophotometry and ultraviolet spectroscopy. The characteristic bands in the infrared spectrum (Figure 1) of verapamil hydrochloride are given in Section 2.1. The ultraviolet spectrum of verapamil hydrochloride is shown in Figure 7.

### 5.2 Elemental Analysis

A typical elemental analysis of a sample of verapamil hydrochloride is presented in Table VI.

Table VI

#### Elemental Analysis of Verapamil Hydrochloride

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
Carbon	66.04	66.32
Hydrogen	8.01	8.08
Nitrogen	5.70	5.72
Oxygen	13.03	12.77
Chlorine	7.22	7.37

### 5.3 Chromatographic Analysis

#### 5.31 Thin-Layer Chromatography

Several thin-layer chromatographic systems have been used to induce the migration of the compound. The thin-layer chromatographic systems are summarized in Table VII.

Table VII  
Thin-Layer Chromatographic Systems  
of Verapamil Hydrochloride

<u>System No.</u>	<u>Solvent System</u>	<u>Adsorbent</u>	<u>Visualization Agent</u>
1.	tert-Butyl Alcohol: sec-Butyl Alcohol: Glacial Acetic Acid: Water(20:50:7:25)	Silica Gel 60	Ceric Sulfate-Molybdate Spray ( $R_f = 0.37$ )
2.	Toluene:Methanol: Acetone:Glacial Acetic Acid (70:20:5:5)	Silica Gel 60	Iodine Vapor or Dragendorff Spray ( $R_f = 0.27$ )
3.	Cyclohexane Diethylamine (85:15)	Silica Gel 60	Spray - Ferric chloride hexahydrate, 5g and iodine, 2g in a mixture of 50 mL of acetone and 50 mL of tartaric acid solution (1 in 5) ( $R_f = 0.25$ )
4.	Methylenechloride: Acetone:Diethylamine (80:15:5)	Silica Gel 60	Iodine Vapor or Dragendorffs Spray ( $R_f = 0.66$ )

#### 5.32 High Performance Liquid Chromatography

Several high performance liquid chromatographic (HPLC) systems have been used to determine the purity of verapamil hydrochloride. The following are typical chromatographic conditions for the HPLC determination of verapamil hydrochloride.

System I

Mobile phase: 0.2 M pH 7.5 phosphate  
buffer:water:acetonitrile:methanol  
(1:26:40:33).  
Column: A Zorbax ODS, 4.6 mm i.d.  
x 25-cm column  
Flow Rate: 1.8 mL/minute  
Elution time: Approximately 8 min.

System II

Mobile Phase: Dissolve 3 g of sodium dodecyl  
sulfate in 360 mL of distilled  
water and 140 mL of methanol, and  
then dilute with acetonitrile to 1  
liter. Adjust the solution with  
perchloric acid to pH 2.5.  
Column: A Zorbax ODS, 4.6-mm i.d. x 25-cm  
column  
Flow Rate: 1.5 mL/minute  
Elution time: Approximately 14 min.

System III

Mobile Phase: 0.1 M pH 3.0 phosphate buffer:  
acetonitrile (60:40)  
Column: A  $\mu$  Bondapak C<sub>18</sub>, 4.6-mm i.d. x  
30-cm column.  
Flow Rate: 2 mL/minute  
Elution Time: Approximately 4 min.

5.4 Titrimetry5.4.1 Perchloric Acid Titration

Verapamil hydrochloride exhibits basic properties. It can be potentiometrically titrated with standardized 0.1 N perchloric acid using a modified glass-calomel electrode system, in which 0.1 N lithium perchlorate in acetic acid has been substituted for potassium chloride, employing glacial acetic acid as the sample solvent and in the presence of mercuric acetate. Also, it can be



titrated with standardized 0.1 N perchloric acid using 1-naphtholbenzene solution as the indicator (12).

#### 5.42 Silver Nitrate Titration

The chloride ion of verapamil hydrochloride can be titrated with standardized 0.1 N silver nitrate solution using a glass-silver (Ag) electrode system and employing water as the sample solvent.

### 6. Analysis of Pharmaceutical Formulations

Verapamil hydrochloride injectable solution and tablet formulations can be analyzed by a high performance liquid chromatography procedure using triphenylene as the internal standard. The method uses a Zorbax ODS column and 0.2 M pH 7.5 phosphate buffer:water:acetonitrile:methanol (1:26:40:33) as the eluent. Injectable solutions have been analyzed by a C<sub>18</sub> micro-Bondapak column and 0.1 M pH 3.0 phosphate buffer - acetonitrile (60:40) as the eluent (13).

### 7. Drug Metabolism and Pharmacokinetics

The metabolism of verapamil has been studied by qualitative and quantitative examination of the 48-hour pooled urine of four subjects who received 80 mg (80  $\mu$ Ci) <sup>14</sup>C-verapamil in 100 mL of water (14). N-Dealkylation is the main metabolic pathway of verapamil, and it yields a secondary amine (22%) and primary amine (3-4%). The N-demethylated product, norverapamil is 6% of the urinary metabolites collected in 48 hours. The O-demethylated products of all these compounds represent about 16-17% of the dose administered and are excreted exclusively as inactive conjugates. The metabolic pathways in the dog and rat have been reported by McIlhenny (15), and are similar to those reported in humans (14).

The pharmacokinetics of verapamil was first reported by Schomerus (16). The results indicated half-lives ranging from 2.7-7.4 hours using a mass fragmentographic analysis. Later studies confirmed these data and found average half-lives of 4.2 hours and 2.0

hours, using a fluorometric method (17) or GC method (18), respectively. The apparent volume of distribution ranges from about 2.5 liters/kg (17,18) to 5.0 liters/kg (16). Verapamil is approximately 90% bound to plasma protein (16); however, this binding is not concentration-dependent over the range of 10-2000 ng/mL (19). The gastrointestinal absorption of orally administered verapamil was estimated from the excretion of  $^{14}\text{C}$ -verapamil. The six healthy volunteers studies indicated that the absorption averaged over 90% (16). The absolute bioavailability in the subjects receiving both the oral and intravenous formulation was about 10-20% (16, 17). The low bioavailability is due to the rapid biotransformation of verapamil in the liver during its first pass through the portal circulation (19).

#### 8. Determination of Verapamil in Biological Fluids

Verapamil in biological fluids and tissues has been analyzed by a spectrofluorometric technique (21). Spiegelhalder and Eichelbaum have used the mass fragmentographic procedure to determine verapamil in human plasma (22). Gas chromatographic determination of verapamil in plasma and urine have been reported by several authors (23,24).

Several high-performance liquid chromatographic procedures have been published for measuring the concentration of verapamil and its active metabolite, norverapamil, in plasma and blood (25-29). The spectrofluorometric method is not specific in that not only verapamil but all fluorescent metabolites are detected. Although the mass fragmentographic procedure is adequately sensitive and specific, it is not applicable for routine monitoring of large numbers of samples due to its complexity and the need for highly sophisticated equipment. The GC and HPLC assay procedures are sensitive for plasma levels of 1-5 ng/mL verapamil. The HPLC technique permits simultaneous determination of verapamil and its major active metabolite, norverapamil (14,30).

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# **POTASSIUM PENICILLIN V**

**David H. Sieh**

## POTASSIUM PENICILLIN V

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The following supplement contains updated information pertaining to the analytical chemistry of potassium phenoxymethylpenicillin. The literature survey is complete up to January 1985. The analytical profile for potassium benzylpenicillin has been published recently<sup>1</sup>.

## 1. Description

### 1.13 Chemical Abstracts Registry Number

The chemical abstracts registry numbers for potassium phenoxymethylpenicillin (potassium penicillin V) and phenoxymethylpenicillin (penicillin V) are 132-98-9 and 87-08-1, respectively.

## 2. Physical Properties

### 2.1 Spectra

#### 2.11 Infrared Spectra

The original profile<sup>2</sup> contains the infrared spectrum of potassium phenoxymethylpenicillin prepared as a potassium bromide pellet. Kuzhelyuk<sup>3</sup> showed through the interpretation of infrared spectra that phenoxymethylpenicillin exists in the acid form, 6-aminopenicillanic acid as a zwitterion and potassium benzylpenicillin (potassium penicillin G) as an ionic structure. An attenuated total reflection method<sup>4</sup> was used to differentiate penicillin V from its potassium and calcium salts in the solid state as a layer of crystals on adhesive tape. The infrared spectra of aqueous antibiotic solutions including potassium penicillin V were obtained by using a Liquid Analyzer accessory in a Fourier-transform IR spectrometer<sup>5</sup>. By measuring the absorbance of the  $\beta$ -lactam carbonyl band, a sensitivity of <0.1% by weight of antibiotic both in aqueous solution and in fermentation broth was realized.

Structural information based on the infrared spectra of penicillin V and its sodium salt allowed Siemon *et al.*<sup>6</sup> to assign a trans conformation to the amide bond of the free acid and a cis conformation to the amide bond of the sodium salt. Infrared spectra of 6-aminopenicillin and several penicillin derivatives including potassium penicillin V were determined and correlated with structure by Dziegielewski *et al.*<sup>7</sup>. Comparison of the solid state infrared spectra of 14 semisynthetic penicillins to penicillin V<sup>8</sup> yielded differences in the stretching vibrations of the carbonyl group in the  $\beta$ -lactam ring, in asymmetric stretching vibrations of the carboxylate ion and in the stretching vibrations of the N-H bond in the peptide portion of the molecule.

Qualitative and quantitative analyses of 11 natural and semisynthetic penicillins including potassium penicillin V was accomplished by Casu and Ventura<sup>9</sup>. The method is based on the inspection of the carbonyl region of the infrared spectrum and on

the measurement of the absorbance of the  $\beta$ -lactam band at about 1760  $\text{cm}^{-1}$ . Steric strain as reflected by the  $\beta$ -lactam carbonyl frequency in 24 penicillins and cephalosporins including penicillin V was correlated with the log of the rate constant for alkaline hydrolysis by Indelicato *et al.*<sup>10</sup>

### 2.12 Nuclear Magnetic Resonance

The improved  $^1\text{H}$ -NMR and the  $^{13}\text{C}$ -NMR spectra<sup>11</sup> of the potassium salt of penicillin V are shown in Figures 1 and 2, respectively (refer to Figure 2, section 2.12 of the original profile<sup>2</sup> for comparison and assignments of the  $^1\text{H}$ -NMR spectrum). The  $^{13}\text{C}$ -NMR spectrum was determined on a Varian XL-100 spectrometer equipped with a Nicolet TT-100 data system. Assignments for the carbon atoms in the  $^{13}\text{C}$ -spectrum are listed in Table I<sup>11</sup>. The assignments are based on relative chemical shifts in PPM from tetramethylsilane with internal reference as dioxane- $\text{d}_4$  = 67.6 PPM and are in agreement with literature values<sup>12,14</sup>.

The  $^1\text{H}$ -NMR spectra of potassium penicillin V and penicillin G determined on a Varian 60 instrument have been used by Wilson *et al.*<sup>13</sup> in identification of the compounds. The natural-abundance  $^{13}\text{C}$ -NMR spectra of penicillin V, its methyl ester, penicillin G and 4 other semisynthetic penicillins have been determined and chemical shifts assigned using pulse Fourier-transform and long-range  $^{13}\text{C}$ -H coupling<sup>14</sup>. Dobson *et al.*<sup>15</sup> used  $^1\text{H}$  and  $^{13}\text{C}$  shift ratios in aqueous solutions of penicillins including penicillin G and penicillin V sulfoxide containing  $\text{Eu}^{3+}$ ,  $\text{Gd}^{3+}$ ,  $\text{La}^{3+}$  and  $\text{Pr}^{3+}$  to determine molecular conformations. In a series of articles, Pek *et al.*<sup>16-18</sup> studied the effects of concentration and solvent on the  $^1\text{H}$ -NMR chemical shifts of the methyl esters of benzyl- and phenoxyethylpenicillins. In addition, the conformation of the HNC(6) fragment was studied by  $^1\text{H}$ -NMR.

### 2.13 Ultraviolet

The ultraviolet spectrum of potassium phenoxyethylpenicillin in methanol determined on a Perkin-Elmer Model 320 spectrophotometer is shown in Figure 3. The maxima occurring at 263, 268 and 275 nm agree well with the data provided in the original profile<sup>2</sup>.

Figure 1.  $^1\text{H}$ -Nuclear Magnetic Resonance Spectrum of Potassium Phenoxyethylpenicillin (SQ19316) in  $\text{D}_2\text{O}$ . (Instrument: Varian XL-100).

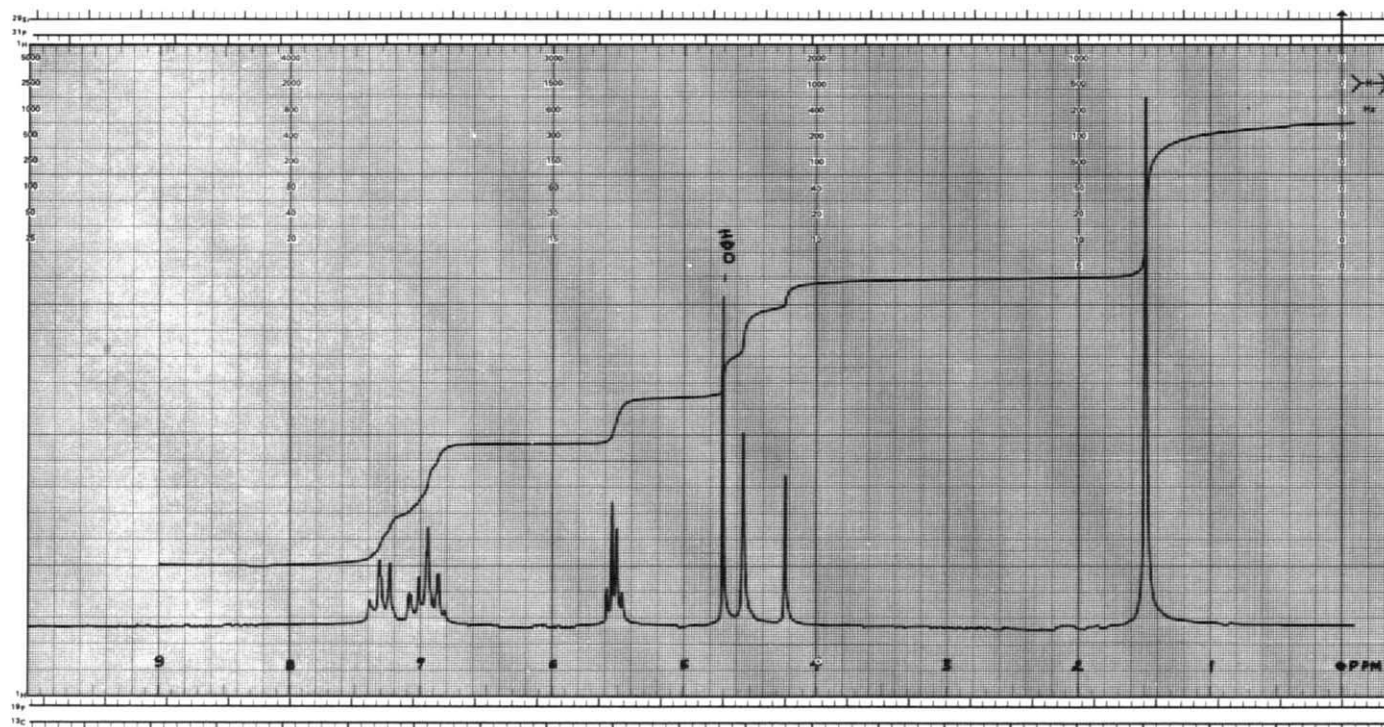


Figure 2.  $^{13}\text{C}$ -Nuclear Magnetic Resonance Spectrum of Potassium Phenoxymethylpenicillin (SQ19316) in  $\text{D}_2\text{O}$ . (Instrument: Varian XL-100).

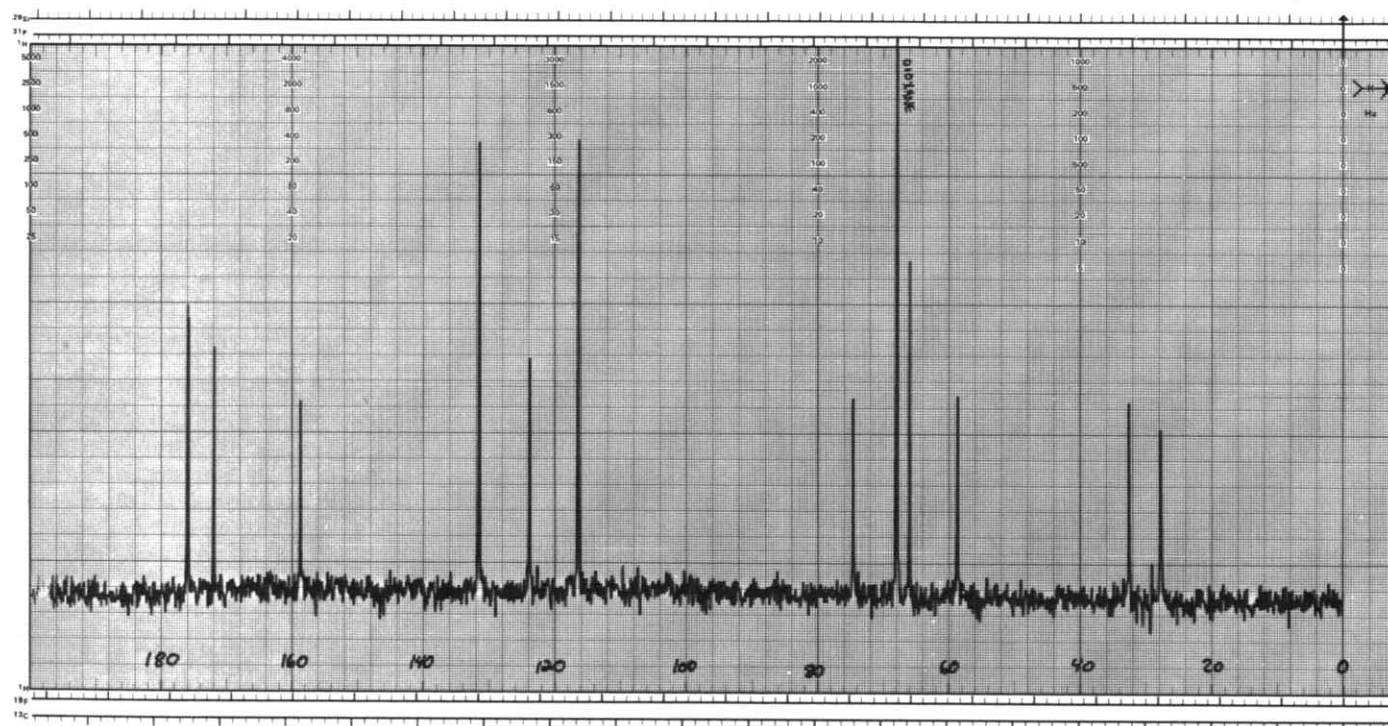
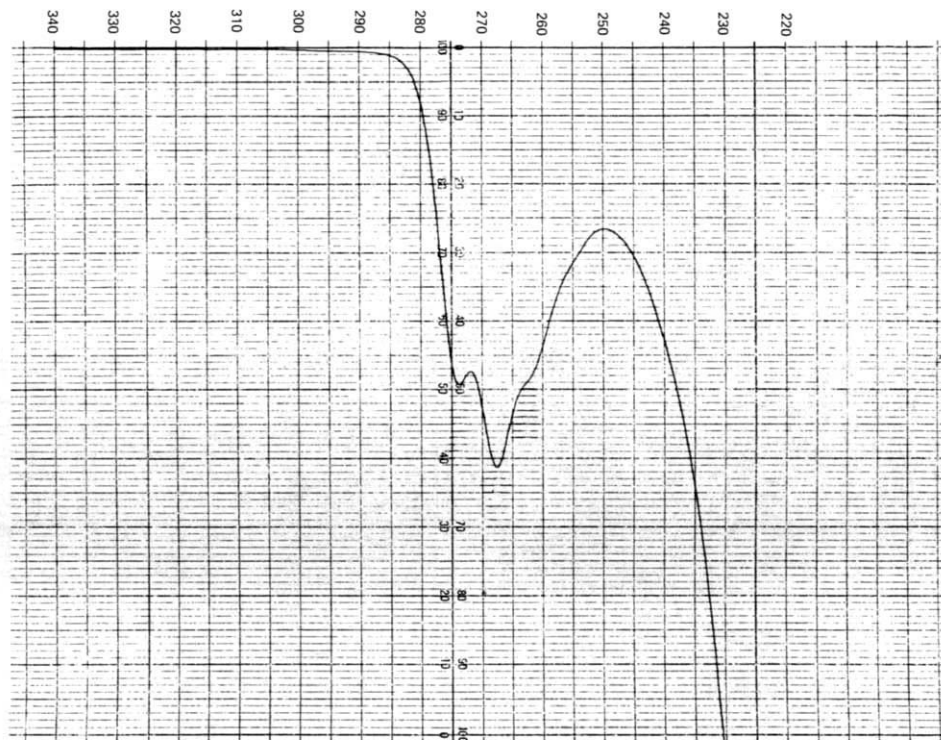


Table 1.  $^{13}\text{C}$ -NMR of Potassium Phenoxymethylpenicillin in  $\text{D}_2\text{O}$ 

<u>Chemical Shift<sup>a</sup></u>	<u>Assignment</u>
27.6	$-\text{CH}_3$
32.4	$-\text{CH}_3$
58.3	$\text{NH}-\underline{\text{C}}\text{H}-\text{CH}-\text{S}$
65.6 <sup>b</sup>	$-\text{OCH}_2-$
65.6 <sup>b</sup>	$>\underline{\text{C}}(\text{CH}_3)_2$
67.6 <sup>c</sup>	$\text{NH}-\text{CH}-\underline{\text{C}}\text{H}-\text{S}$
74.1	$-\underline{\text{C}}\text{H}-\text{COOH}$
115.6	$\phi\text{-O-}(\text{ortho carbons})$
123.0	$\phi\text{-O-}(\text{para carbon})$
130.6	$\phi\text{-O-}(\text{meta carbons})$
157.7	$\phi\text{-O-}(\text{ipso carbon})$
170.9 <sup>d</sup>	$-\text{CO}_2\text{H}$
174.8 <sup>d</sup>	$-\text{CONH}-$
174.9 <sup>d</sup>	$-\text{CON}<$

- 
- a Reference to dioxane @ 67.6 PPM  
 b Overlapping peaks  
 c Overlapped with reference peak  
 d May be interchanged

Figure 3. Ultraviolet Spectrum of Potassium Phenoxymethylpenicillin in Methanol  
(Instrument: Perkin-Elmer Model 320).



## 2.14 Mass Spectra

The fast atom bombardment (FAB) mass spectrum<sup>19</sup> of potassium penicillin V is shown in Figure 4. The top figure is that of positive ions while the bottom figure represents negative ions. Approximately 10 micrograms of analyte was dissolved in 5 microliters of dithiothreitol: dithioerythritol matrix and sputtered with 8 keV xenon atoms. The resulting secondary ions were mass analyzed using a VG-ZAB-2F mass spectrometer. Figure 5 details the fragmentation pattern observed.

Various ionic forms of the parent compound are observed including the  $(M-K)^-$  and the  $(M-H)^-$  ions (Figure 4 - bottom) at  $m/z$  349 and 387. Their positive ion counterparts (Figure 4 - top) are the  $(M+H)^+$ ,  $(M+K)^+$  and  $(M+2K-H)^+$  parents at  $m/z$  389, 427 and 465, respectively. Decarboxylation accounts for the  $305^-$  and the  $345^+$  fragments. The  $77^+$ ,  $93^-$  and  $107^+$  fragments are formed by direct cleavage as shown in Figure 5. The  $153^-$  and  $191^-$  ions are due to the FAB solvent. Most other fragments are generated via retro-Diels Alder cleavage of the beta-lactam ring. The  $236^+$  fragment is the dipotassiated daughter. Oxygen transfer from the carboxyl group to the carbonyl of the beta-lactam ring yields the  $208^-$  and  $246^-$  (monopotassiated form) fragments. Subsequent loss of the elements of phenol from the  $208^-$  fragment yields the  $114^-$  daughter. The less intense  $122^+$  and  $171^+$  daughter ions are also represented in Figure 5.

Attempts to ionize penicillin V by desorption chemical ionization (DCI) and field desorption (FD) were less successful than by FAB. Addition of 1.0N aqueous HCl to the analyte yielded a weak but significant  $(M+H)^+$  parent at  $m/z$  351 by DCI and a molecular ion,  $M^+$ , at  $m/z$  350 by FD of the free acid form<sup>18</sup>.

Mueller *et al.*<sup>20</sup> characterized a number of clinically significant penicillins and cephalosporins including penicillins V and G by pyrolysis mass spectrometry. Major fragments found were  $m/z$  94, 108 and 133. The characteristics of in-beam electron ionization mass spectra of 6-aminopenicillanic acid and several penicillins including penicillins V and G were determined by Ohashi *et al.*<sup>21</sup>. The  $(M-K)^-$  ion of potassium penicillin V was observed by FD mass spectrometry<sup>22</sup>. The penicillin was mixed with polyethylene oxide in water and applied to a tungsten emitter to generate the ion.

Barbalas *et al.*<sup>23</sup> reported a technique for the targeted class analysis of  $\beta$ -lactam antibiotics including penicillin V and its methylester using tandem mass spectrometry. Electron-impact ionization of a  $\beta$ -lactam gave a major fragment ion by cleavage of the lactam ring. Collisional activation dissociation of this ion then gave spectra characteristic of the compound class in terms of ring size and substituent type and position. A combined

Figure 4. Fast Atom Bombardment (FAB) Mass Spectrum of Potassium Phenoxymethylpenicillin (Instrument: VG-ZAB-2F).

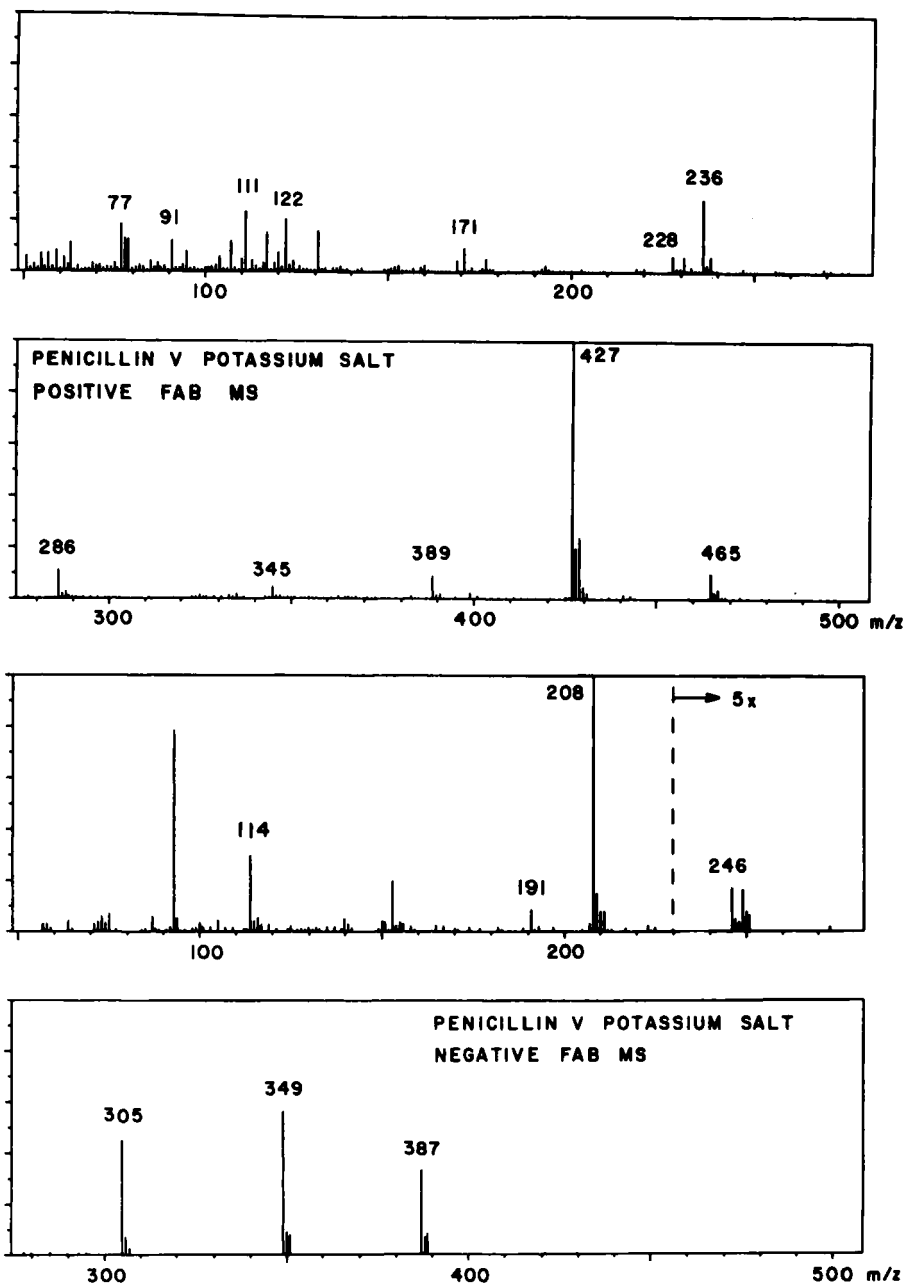
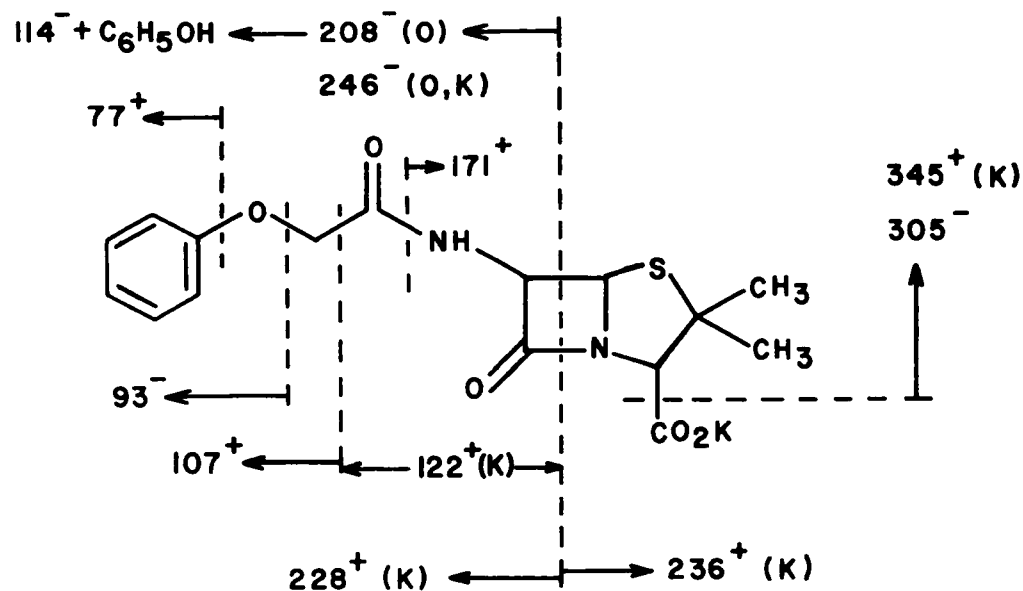




Figure 5. Fragmentation Pattern of Potassium Phenoxymethylpenicillin.



PENICILLIN V POTASSIUM SALT

microbore liquid chromatographic mass spectrometric procedure has been used to analyze mixtures of penicillins such as penicillin G and pseudomonic acids<sup>24</sup>. Penicillin V is quite likely to be amenable to this technique.

## 2.2 Crystal Properties

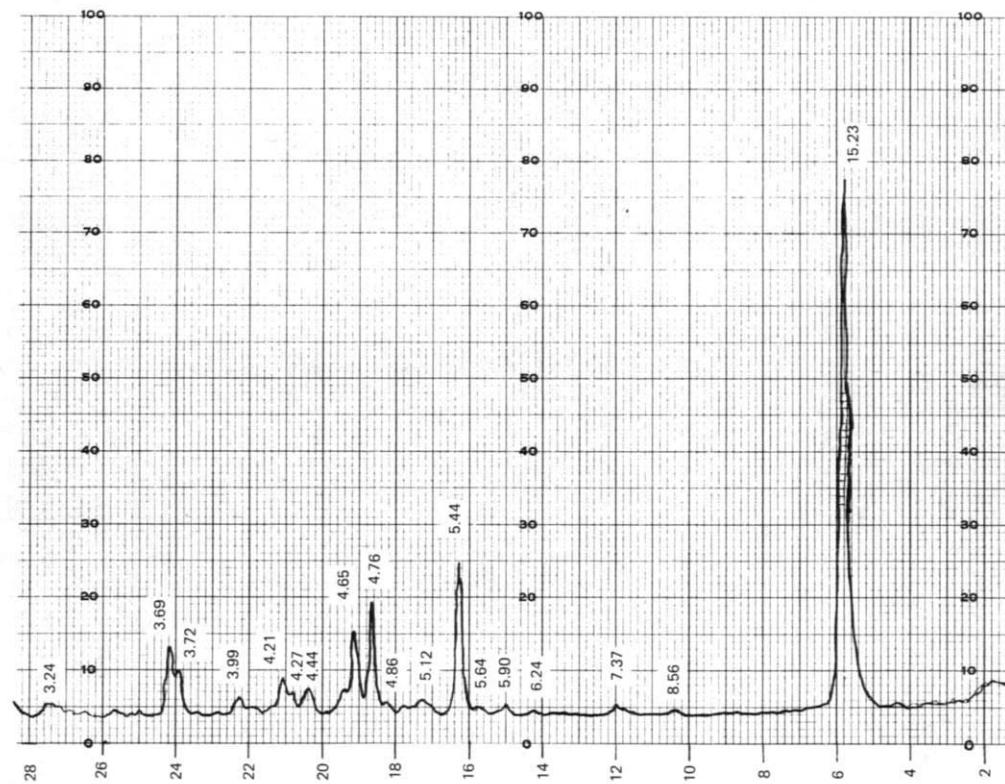
The powder X-ray diffraction pattern of potassium phenoxymethylpenicillin obtained using Cu-K $\alpha$ , nickel filtered radiation is shown in Figure 6<sup>25</sup>. Table II lists the relative intensities using the peak at 15.23 Å° as the reference = 1.00.

Table II. X-ray Diffraction Pattern of Potassium Phenoxymethylpenicillin

<u>2<math>\theta</math> (Deg)</u>	<u>d (Å°)</u>	<u>I/I<sub>o</sub></u>
5.80	15.23	1.000
10.30	8.56	0.014
12.00	7.37	0.014
14.20	6.24	0.014
15.00	5.90	0.027
15.70	5.64	0.014
16.30	5.44	0.284
17.25	5.12	0.027
18.20	4.86	0.027
18.65	4.76	0.203
19.10	4.65	0.149
20.40	4.41	0.054
20.80	4.27	0.041
21.10	4.21	0.068
22.25	3.99	0.027
23.95	3.72	0.081
24.15	3.69	0.122
27.50	3.24	0.014

The single crystal x-ray diffraction pattern and biological activity of phenoxymethylanhydronicillin was compared to phenoxymethylpenicillin by Simon *et al.*<sup>26</sup>. Through analysis of crystal structures the conformation of the thiazolidine ring in ampicillin trihydrate has been compared to penicillins V and G and penicillin V sulfoxide<sup>27</sup>. Ampicillin trihydrate and penicillin V sulfoxide were found to have the S(1) atom out of plane while in penicillins V and G the C(3) atom was out of plane (Table III).

Figure 6. Powder X-ray Diffractogram of Potassium Phenoxymethylpenicillin (Squibb Reference Standard).



**Table III. Conformation of Thiazolidine Rings in Penicillins**

<u>Penicillin</u>	<u>Planar portion formed by</u>	<u>Atom out of plane</u>	<u>Distance of atom from plane (Å°)</u>
Ampicillin Trihydrate	C(2)C(3)N(4)C(5)	S(1)	0.84
Penicillin V	S(1)C(2)N(4)C(5)	C(3)	0.51
Penicillin G	S(1)C(2)N(4)C(5)	C(3)	0.4-0.5
Penicillin V Sulfoxide	C(2)C(3)N(4)C(5)	S(1)	---

Conformation of the thioazolidine ring and its effect on biological activity has been extensively studied. Virudachalam *et al.*<sup>28</sup> found that  $\beta$ -lactam antibiotics such as penicillin V assume a conformation similar to X-D-alanyl-D-alanine due to the presence of the lactam ring. Substitution of CH<sub>3</sub> for the 6a or 7a H completely inactivated penicillins.

Empirical potential energy calculations were carried out by Vasudevan and Rao<sup>29</sup> to determine the preferred conformations of penicillins, including penicillin V, and their sulfones. They found that the C(3) puckered conformation in penicillins like penicillin V was favored. Replacement of the C(2) methyl groups by H atoms, as in penicillin V sulfone, made the C(3) puckered conformation much less favorable. In penicillins in which the C(3) atom was out of the plane of the thiazolidine ring, i.e., penicillins V and G, a narrow antibacterial range was found<sup>30</sup>. Alternately, when the S(1) atom was out of plane, a broad antibacterial range was found. The basis for the broader antibacterial range was postulated to be due to a closer proximity of the carboxyl to the carbonyl moiety.

A series of 10 penicillin sodium salts including sodium penicillin V and G was analyzed by thermoanalytical techniques i.e., thermogravimetry (TG), differential thermogravimetry (DTG) and differential scanning calorimetry (DSC)<sup>31</sup>. The analyses were carried out in an oxygen atmosphere. DSC and DTG curves are given. Ammonium sulfate was added for better accuracy and precision.

One of the parameters used to measure the acceptability of powders is friability. Friability is a measure of the powders ability to crumble and is usually measured in grams per second. Ezerskii<sup>32</sup> found the friability of potassium penicillin V powder as was most antibiotic powders to be  $< 3.15$  g/sec.

A microencapsulation technique used in the recrystallization of potassium penicillin V has been described by Fukushima and Sasagawa<sup>33</sup>. Crystalline potassium penicillin V was suspended in n-butanol, added to an acetone solution of encapsulating agent (Eudragit S), stirred at room temperature to evaporate as much acetone as possible and then petroleum ether was added. A method for growing single crystals of penicillin V and its sulfoxide as the crystal falls freely through a rotating liquid was reported by Tensmeyer et al.<sup>34</sup>.

## 2.3 Solution Properties

### 2.31 Optical Properties

Lisowski et al.<sup>35</sup> determined the optical rotatory dispersion (ORD) and circular dichroism (CD) spectra of penicillin V, its methyl ester and its sulfoxide and methyl ester of the sulfoxide. The penicillin system was characterized by a strong positive Cotton effect at 233 nm due to  $n \rightarrow \sigma^*$  excitation of the sulfide chromophore. ORD and CD spectra of potassium penicillin V and 10 other penicillins were reported by Mitscher et al.<sup>36</sup>. Analysis of the spectrum of potassium penicillin V yielded values of  $3.07 \times 10^{-3}$  @ 244 nm and  $3.31 \times 10^{-4}$  @ 230 nm for  $(\phi)$  max and  $(\theta)$  max, respectively, in 0.1M pH 6.5 citrate buffer. The results when compared with published X-ray data suggest that some conformational changes can apparently go undetected by chiroptical techniques.

The orthogonal function method was extended to correct for interferences in spectropolarimetric analysis and was applied to the determination of potassium penicillin V and potassium penicillin G in the presence of their degradation products (penicilloic acids)<sup>37</sup>. Polarimetric data are tabulated for penicillin V and 12 other antibiotics by Avico et al.<sup>38</sup>. Optical rotatory changes elicited by enzymatic catalysis were used to detect losses in drug activity of phenoxymethyl- and benzylpenicillin in solution at room temperature<sup>39</sup>.

### 2.32 Partition Coefficients

The partition coefficients of phenoxymethylpenicillin in various systems are shown in Table IV. The solubility of many drugs including penicillin V in water has been estimated based on the correlation of partition coefficients in an octanol-water system and hydrophilicity and lipophilicity<sup>40</sup>. Yamana et al.<sup>41</sup> used high performance liquid chromatography for determining

partition coefficients of 17 penicillins including V and G and cephalosporins. Apparent partition coefficients of 12  $\beta$ -lactam antibiotics were determined in octanol-water and 2-methyl-propanol-water systems at various pH values by Tsuji *et al.*<sup>44</sup>. Using these values with potentiometric analytical data, the pK for penicillin V was calculated to be 2.79 at 37°C, which correlated well with the previously published literature values of 2.73 and 2.74 at 25°C<sup>45</sup>.

**Table IV. Partition Coefficients of Phenoxyethylpenicillin**

<u>System</u>	<u>Form</u>	<u>log P<sup>a</sup></u>	<u>Ref.</u>
octanol/H <sub>2</sub> O	Free acid	2.1 (2.4)	39
octanol/H <sub>2</sub> O	Free acid	2.01(2.05)	40
isobutanol/H <sub>2</sub> O	Free acid	1.10	41
isobutanol/ aqueous buffer	Free acid	20	42
isobutanol/ aqueous buffer	Anion	0.90	42

<sup>a</sup>Calculated value in parentheses

Distribution of penicillins V and G in butyl acetate-water-potassium carbonate followed by extraction at pH 7 has been studied by Russian workers<sup>46-48</sup>. Equations were also derived for calculating the mass transfer coefficient, K, of undissociated penicillin V from butyl acetate to aqueous buffers. The value of K increased with the rate of mixing of the phases, with interphase tension and with decreasing viscosity of the aqueous phase.

### 2.33 Ion-pairing and Hydrogen-bonding

Penicillins such as penicillin V can form adducts with many different types of compounds. This property allowed Schroder-Nielsen<sup>49</sup> to extract penicillin V as a 1:2 adduct with trioctylphosphine oxide from water by chloroform or hexane. Other studies in these laboratories<sup>50,51</sup> showed that penicillin V could be extracted from an aqueous phase to an organic phase as ion pairs with octylamine, trimethylnonylammonium hydroxide, tetrabutylamine and n-dodecylamine.

The extraction of penicillin V and its precursor phenoxyacetic acid with 1°, 2° and 3° amines at various pH values

was reported by Reschke and Schuegerl<sup>52</sup>. Through the formation of a 1:1 adduct with riboflavin 2',3',4',5'-tetraacetate, penicillin V was found to be an effective quencher of the fluorescence of the former compound<sup>53</sup>. The author suggests that the quenching appears due mainly to coplanar interaction through hydrogen bonding.

### 2.34 Metal Complexation

Using a pH-titration technique in 50% aqueous acetone at 20-40°C at an ionic strength of 0.1 (NaClO<sub>4</sub>), the complexation of Ag(I), Pb(II), Zn(II), Be(II), Ca(II), Mg(II), Mn(II), Fe(III), Al(III) and Cr(III) with penicillin V was studied extensively by Tiwari *et al.*<sup>54,55</sup> and Chakrawarti *et al.*<sup>56,57</sup>. Thermodynamic parameters of the complexation reaction and stability constants of the complexes were evaluated. High negative enthalpies obtained for the complexation reaction with Be(II), Mg(II) and Ca(II) indicate a considerable degree of covalence in the metal to ligand bond. Stability constants for the complexation of penicillin V with Eu(III) and Gd(III) in 50% aqueous acetone at 0.1M ionic strength at 25°C were reported to be 9.68 and 9.63, respectively by Sawhney and Dangwal<sup>58</sup>.

### 2.35 Miscellaneous

The kinetics and dynamics of the sorption and desorption of penicillin V were studied using ion exchangers by Vedeneeva *et al.*<sup>59</sup>. Fluorescence studies<sup>60</sup> of 6 semisynthetic penicillins including phenoxymethylpenicillin in dilute solution showed various interactions between side-chain and heterocyclic rings. In penicillins where the exocyclic amide is  $\alpha$ -substituted with an electron withdrawing group, increased acid stability correlated well with the observed molecular conformation. The aggregation of a number of penicillins including penicillin V both in water and in electrolyte solution was examined by total intensity light scattering methods<sup>61</sup>. Micellar association was noted for penicillin V and critical micelle concentrations and micellar aggregation numbers were determined.

## 3. Stability and Degradation

### 3.1 Stability

#### 3.11 Temperature Effects

The effect of freezing on the stability of potassium penicillin V solutions was studied by Allen and Lo<sup>62</sup>. Potassium penicillin V was found to retain at least 90% of its activity after 60 days of storage at -10°C. In addition, degradation was shown to be first order. Other investigators<sup>63-64</sup> have studied the effects of temperature on the stability of potassium penicillin V in solution and concluded that the decomposition follows first order kinetics.

During investigation of the lower limit of ignition of aerosuspensions, Yashin *et al.*<sup>65</sup> found that potassium penicillin V underwent an exothermic transformation at 170°C with vigorous evolution of gas. Trial batches of potassium penicillin V showed excellent biostability and activity over 4 years at -20,33 and 50°C<sup>66</sup>.

### 3.12 Photostability

Irradiation of potassium phenoxymethylpenicillin may lead to decomposition depending on the type and intensity of the light source. Table V summarizes data found in the literature. In general, the order of photostability is potassium penicillin V > penicillin V > penicillin G.

Table V. Photostability of Penicillin V

<u>Source</u>	<u>Physical Form</u>	<u>Destructive Effect</u>	<u>Comment</u>	<u>Ref.</u>
Xenon	pH 4, 8	None	52 antibiotics studied	67
Ultraviolet	solution	None	NaPen G decomposed	68
Gamma	solid, solution	Slight	Radiation yields and radiolysis products studied	69
Gamma	solution	Slight	Decomposition proportional to radiation dose	70
Gamma, neutron	solution	Slight	Na <sub>2</sub> S <sub>2</sub> O <sub>7</sub> not a stabilizer	71



### 3.13 Surfactants

Interaction between  $\beta$ -lactam antibiotics and surfactant micelles and their effects on aqueous stability and solubility were investigated by Tsuji *et al.*<sup>72,73</sup> They observed large differences in binding constants between the undissociated and ionized forms of penicillins such as penicillin V from the interaction with nonionic and anionic micelles of polyoxyethylene-23-lauryl ether and sodium lauryl sulfate. However no significant difference in the binding constants for either form was observed with a cationic surfactant, cetyltrimethylammonium bromide. Acid degradation of penicillin V was minimized in nonionic and cationic surfactants but was facilitated by anionic surfactants. The solubility of penicillin V was increased threefold in the presence of polyoxyethylene-23-lauryl ether. Doncheva *et al.*<sup>74</sup> reported the stabilization of a suspension of phenoxymethylpenicillin by surfactants i.e., Tweens, Myrjs, etc.

### 3.14 Sucrose

The kinetics of degradation of a number of semi-synthetic penicillins including potassium penicillin V in aqueous sucrose solutions over a pH range from 6 to 10 was investigated by Bundgaard and Larsen<sup>75</sup>. They observed that sucrose accelerated the rate of hydrolysis of penicillins to penicilloic acids entirely through a nucleophilic pathway with an intermediate formation of penicilloyl sucrose esters. Hem *et al.*<sup>76</sup> had previously found that sucrose formed 1:1 complexes with several penicillins including potassium penicillin V and that the rate of degradation of complexed penicillin was 5-6 times the rate for uncomplexed penicillin. No change in degradation pathway was noted. However, Wellmann *et al.*<sup>77</sup> reported that the accelerating effect of sucrose was not based on complexation but rather the formation of an additional degradation product in sugar solutions. The product, probably the penicilloyl ester, was hydrolyzed to sugar and penicilloic acid.

### 3.15 Miscellaneous

Ferrero<sup>78</sup> used a manometric method to determine the stability of various penicillins on exposure to intestinal microflora from rats. Phenoxymethylpenicillin and methicillin were found to be highly susceptible to inactivation by bacteria with less than 10% of the starting concentration remaining after 4 hours. Differential thermal analysis was used to reveal drug instability during storage of potassium penicillin V and PEG 20,000<sup>79</sup>. Harwood *et al.*<sup>80</sup> found that in the presence of Cu(II)-glycine chelates, penicillins including penicillin V are quantitatively hydrolyzed to penicilloic acid. This is in direct contrast to the much slower reaction between penicillin and glycine in the

absence of Cu(II), in which the major degradation product is the penicilloamide.

### 3.2 Degradation

#### 3.21 Enzymatic

The enzymatic hydrolysis of penicillins has been reviewed by Carrington<sup>81</sup>. Numerous isolated enzymes and media have been found to hydrolyze penicillin V (Table VI). In most of the studies, the nature of the penicillin side chain, particularly the stereo-specificity, played a significant role in the stability of the molecule toward the enzymes.

The effect of ionic strength, temperature and pH on the kinetic behavior of the staphylococcal and bacillary  $\beta$ -lactamase catalyzed hydrolysis of penicillins containing various side chain groups and nucleus structures including penicillin V was studied by Hou and Poole<sup>94</sup>.

The kinetics of penicillin V deacylation on an immobilized enzyme was studied by Haagensen *et al.*<sup>96</sup>. The reaction rate was found to depend strongly on pH and both products, phenoxy-acetic acid and 6-aminopenicillanic acid, inhibited the reaction. Gestrelus<sup>97</sup> described the development and properties of a commercially available non-thiol-containing immobilized penicillin V acylase. It can be used to produce 6-aminopenicillanic acid continuously from penicillin V in a series of packed bed reactors with intermediary pH regulation.

The logarithm of the rate constant for penicillinase hydrolysis of a series of phenoxymethylpenicillins substituted on the benzene ring has been correlated with Hammett  $\sigma$  constants<sup>95</sup>. Electron-donating substituents i.e., methyl, ethyl increased the antimicrobial activity while electron-withdrawing substituents such as Cl or NO<sub>2</sub> reduced antimicrobial activity.

#### 3.22 Alkaline degradation

The major product resulting from mild alkaline hydrolysis of penicillins is the dibasic penicilloic acid. Alkaline hydrolysis of penicillin V is usually first order with respect to penicillin but at pH > 10. Garcia de la Pena<sup>98</sup> found that the hydrolysis was catalyzed by OH<sup>-</sup> and was second order. The rate of attack on the anion was seven times greater than that on the undissociated acid.

The catalytic effects of mono- and dihydrogen phosphate ions on the alkaline degradation of penicillins V and G were reported by Finholt<sup>99</sup>. The catalytic constants for penicillin V were calculated to be 0.1 hr<sup>-1</sup> mole<sup>-1</sup>L and 6.2 hr<sup>-1</sup> mole<sup>-1</sup>L for H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and HPO<sub>4</sub><sup>2-</sup>, respectively. A general method was described by Hempenstall *et al.*<sup>100</sup> for the determination of the kinetics of nonisothermal degradation at alkaline pH of potassium penicillin V using a microcomputer.

Table VI. Enzymatic Hydrolysis of Penicillin V

<u>Medium</u>	<u>Specificity</u>	<u>K<sub>M</sub></u>	<u>Comment</u>	<u>Ref.</u>
<i>N. crassa</i>	non-specific	--	A	85
<i>Streptomyces</i> R61	non-specific	--	B	86,87
<i>Bacillus cereus</i> 569/H	$\beta$ -lactamase	64 mM	--	88
<i>S. aureus</i>	$\beta$ -lactamase	--	--	90
<i>E. coli</i> K-12	$\beta$ -lactamase R-TEM	21 $\mu$ M	--	93
<i>F. semitectum</i>	penicillin V acylase	4.75 mM	--	82
<i>E. aroideae</i>	penicillin V acylase	--	--	83
<i>B. plumbea</i>	penicillin V acylase	1.67 mM	C	84
<i>B. megaterium</i>	penicillin V acylase	--	--	89
<i>S. venezuela</i>	penicillin V	--	--	89
<i>B. plumbea</i> NRRL 3824	penicillin V acylase	--	D	91
<i>E. coli</i> 5K	penicillin V acylase	--	--	91
<i>E. aroideae</i>	penicillin V acylase	31 mM	--	92

- 
- A Penicillin V hydrolyzed more slowly than penicillin G
- B Reference 86: 5-<sup>14</sup>C-penicillin V exposed to exocellular DD-carboxypeptidase-transpeptidase:  
Reference 87: Isotopic studies of the mechanism of H fixation on C(6) using D<sub>2</sub>O
- C Maximum activity at 52°C and pH 7.5.  
Activation energy = 16.45 kJ/mole
- D Homogenized medium

Substituents can have a profound effect on chemical reactivity. However, 6  $\alpha$  substitution of penicillin V with  $-\text{CH}_3$ ,  $-\text{OCH}_3$ , or  $-\text{SCH}_3$  gave compounds with only slightly less reactive  $\beta$ -lactams<sup>101</sup> than the parent compound to alkaline hydrolysis. The reduction in reactivity was postulated to be due to steric rather than polar effects. Indelicato *et al.*<sup>10</sup> also correlated the log of the rate constant for alkaline hydrolysis with  $\beta$ -lactam IR carbonyl frequency. Modification of the acylamido side chain resulted in insignificant change in  $\beta$ -lactam reactivity.

### 3.23 Acidic or Neutral

In the original profile<sup>2</sup> (section 3.1, Figure 3), a schematic representation of penicillin degradation is presented. This figure will not be repeated in the present profile, however, a few salient features will be mentioned. Penicillin is hydrolyzed in acidic media to penillic acid. At neutral pH, degradation to penicillenic acid then penicilloic acid occurs. The penicilloic acid further degrades in acidic media to penilloic acid. Reaction of  $\text{Hg(II)}$  and penicilloic acid produces penaldic acid and penicillamine. This degradation forms the basis for sensitive analytical methods for penicillins V and G (See Section 6).

Penicillin V is much more acid stable than penicillin G. Using UV absorption and fluorescence spectra of several semisynthetic penicillins, including penicillins V and G, Assenmacher<sup>102</sup> deduced that penicillin G had the side chain folded in the direction of S, leaving the  $\beta$ -lactam moiety unprotected. Penicillin V on the other hand had the side chain folded in the direction of the  $\beta$ -lactam moiety providing protection and is, therefore, more acid stable. Hartmann *et al.*<sup>103</sup> found that in acidic and neutral aqueous solutions, potassium phenoxymethylpenicillin was hydrolyzed to phenoxymethylpenicilloic acid,  $\alpha$ -phenoxy-methylpenilloic acid,  $\beta$ -phenoxy-methylpenilloic acid and D-penicillamine. The pH-hydrolysis rate curve had a minimum at pH 5.7.

In strongly acidic media the rate of decomposition of penicillin V is considerably faster. For example at pH 1.3, Zakrzewski *et al.*<sup>104</sup> reported that a 50% solution of potassium penicillin V completely degraded in 45 minutes. Jansholt *et al.*<sup>105</sup> found that the degradation of penicillin V was first order with  $k = 0.0301 \text{ min}^{-1}$  at pH 1,  $k = 0.0045 \text{ min}^{-1}$  at pH 2 and  $k = 0.00117 \text{ min}^{-1}$  at pH 3. Half lives of penicillins V and G in 0.067 N HCl at 37°C (simulated conditions in the human stomach) were found by Dusinsky<sup>106</sup> to be 2 hours and 2 minutes, respectively.

As in base hydrolysis, correlation between reaction rate constants for the hydrolysis of penicillin V in acidic media and the Hammett equation has been accomplished<sup>107</sup>. Hydrolysis was facilitated by electron-donating substituents and decreased by electron withdrawing ones.

#### 4. Synthesis and Reactions

##### 4.1 Biosynthesis and Fermentation

An excellent review of the biosynthesis of  $\beta$ -lactam antibiotics is presented by Queener and Neuss<sup>108</sup>. A thorough discussion of steps common to the biosynthesis of penicillins and cephalosporins and the enzymatic conversion of isopenicillin N to *Penicillium*-type penicillins such as penicillins V and G is given. Several earlier reviews<sup>81,109-110</sup> also contain valuable information on the biosynthesis of penicillins.

The biosynthetic pathways by which penicillins are formed have been studied with the use of labelled amino acid intermediates. The two key amino acids involved in penicillin formation are cysteine and valine. In fact, the tripeptide  $\delta$ -(L- $\alpha$ -aminoadipyl)-L-cysteinyl-D-valine has been isolated from the mycelium of *P. crysogenum*, a mold that produces penicillin, *C. acremonium* and *Streptomyces clavuligerus*<sup>110</sup>.

Through labeling experiments Aberhart *et al.*<sup>111,112</sup> showed that (2R,3S)-valine-4-<sup>13</sup>C and DL-valine-Me<sub>2</sub>-d<sub>6</sub> were incorporated into penicillin V by *P. crysogenum*. Mass spectrometry and <sup>13</sup>C-NMR were used as the analytical finish. Oxygen-18 labeling experiments allowed Delderfield *et al.*<sup>113</sup> to show that L-valine-<sup>18</sup>O<sub>2</sub> was incorporated into Penicillin V by *P. crysogenum* with the elimination of 1 of the 2 carboxy O atoms.

*P. crysogenum* incorporates many monosubstituted acetic acids into penicillins so that the acid becomes the acyl substituent attached at the C-6 nitrogen in the penicillin via an amide linkage e.g., incorporation of phenoxyacetic acid leads to penicillin V and phenylacetic acid to penicillin G. Many different methods of fermentation for the production of penicillin V by *P. crysogenum* exist, mainly in the patent literature (Table VII).

The production of penicillin has been improved by sporulation of *P. crysogenum* in p-fluorophenylalanine<sup>126</sup>, addition of nitrofurazone to the culture medium<sup>126</sup>, irradiation of the enzyme with a sublethal dose of UV light<sup>374</sup> or addition of an antioxidant such as di-t-butyl-4-methylphenol<sup>127</sup>. Addition of penicillin V at any time during penicillin V or G fermentation inhibited further accumulation of the antibiotic<sup>128</sup>.

Control of penicillin fermentation has been achieved through the use of penicillin-sensitive electrodes<sup>129</sup>. Broth was removed, the electrode response determined and a known amount of penicillin added to calibrate the electrode. An oxygen-sensitive electrode was used by Squires<sup>130</sup> to study the regulation and control of penicillin V fermentation. The output of the electrode was connected to a pneumatic controller that regulated the rate of continuous sugar addition to the fermentor.

Analysis of the <sup>13</sup>C-NMR spectrum of a concentrated broth from *P. crysogenum* fermentation revealed the presence of

penicillin V and 6-oxopiperidine-2-carboxylic acid as the principal constituents<sup>131</sup>. The lactam isolated from the broth was nearly racemic. Penicillins V and G have been interconverted by *P. crysogenum* through careful control of reaction conditions<sup>132</sup>.

#### 4.2 Separation and Purification

Generally, penicillin V is recovered and purified from fermentation broth by physical means<sup>133,134</sup>. Phenoxyacetic acid<sup>135</sup> and phenoxymethylpenicilloic acid<sup>136</sup> have been preferentially removed by a series of extractions, filtration and precipitation. Addition of N,N'-dibenzylethylenediamine diacetate in ethanol: butyl acetate (1:1) to an aqueous solution of penicillin V and subsequent recrystallization allowed Ege<sup>137</sup> to purify penicillin V. A similar technique, where guanidine compounds were added to aqueous mixtures of penicillin V or G, enabled Cieslak *et al.*<sup>138</sup> to isolate and purify potassium phenoxymethylpenicillin.

Nonextractive penicillin V recovery processes from fermentation broths have been the subject of two patents<sup>142,143</sup>. The salient features are multiple adjustments of pH, several recrystallization steps and addition of potassium 2-ethyl hexanoate to form the potassium salt of penicillin V. Another nonextractive process was published by Kennedy and McCormick<sup>144</sup>. In this method, 1-hexanol and sulfuric acid are added to the broth and then the solvent is evaporated azeotropically at 35°C under reduced pressure. Saturated potassium acetate is added to the filtrate and product recovered.

Ultrafiltration of culture media through semipermeable membranes of poly(1,3,4-oxadiazole) allowed Russian workers<sup>143,144</sup> to remove higher molecular weight protein impurities and colored compounds from penicillin V in fermentation broth. Adsorption of penicillin V on a resin comprised of a nonionic macroporous copolymer of styrene divinylbenzene followed by elution with an organic solvent provided an efficient recovery of relatively pure penicillin V<sup>139</sup>.

#### 4.3 Chemical Synthesis and Reactions

Good reviews of the methodology for penicillin synthesis have been published<sup>108,145</sup>. Penicillin V has been prepared by reaction of 6-aminopenicillanic acid with the phenylglycine derivative of phenoxyacetic acid in an ethanolic solution of N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinone<sup>146</sup>. Heuser<sup>147</sup> extracted a penicillin G hydrolyzate at pH 3.5 with a solution of salicylaldehyde in methylcyclohexanone to give the Schiff base followed by acylation with benzoyl chloride to afford penicillin V. Penicillin V has also been prepared by treating 6-amino-penicillanic acid or its esters with nitriles followed by hydrolysis<sup>148</sup>.

Table VII. Biosynthesis of Penicillin V by Fermentation with *Penicillium crysogenum*.

<u>Strain</u>	<u>Carbon Source</u>	<u>Nitrogen Source</u>	<u>Other Additives</u>	<u>Side Chain Precursor</u>	<u>Yield (units/ml)</u>	<u>Ref.</u>
S3723	glucose + organic acid	--	--	PAA <sup>1</sup>	--	114
ATCC 20168	paraffin (C <sub>12</sub> -C <sub>14</sub> )	--	--	PAA	4825 <sup>2</sup>	115
UV mutant	sucrose	--	--	PAA	11,424 <sup>2</sup>	116
--	corn steep + sucrose	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	--	PAA	14-15,000 <sup>2</sup>	117
--	soybean meal	--	ethanol	PAA	9-10,500 <sup>2</sup>	118
--	corn steep + sucrose	peanut meal	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , CaCO <sub>3</sub>	PAA	4920 <sup>2</sup>	119
--	corn steep + lactose	--	inorganic salts	phenoxyalkanes (C <sub>7</sub> -C <sub>14</sub> )	--	120
S3723	glucose + acetic acid	--	--	PAA	--	121
LAR 0190	sucrose + sunflower oil	--	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , CaCO <sub>3</sub>	PAA	12,330 <sup>2</sup>	122

Table VII., continued:

<u>Strain</u>	<u>Carbon Source</u>	<u>Nitrogen Source</u>	<u>Other Additives</u>	<u>Side Chain Precursor</u>	<u>Yield (units/ml)</u>	<u>Ref.</u>
--	sucrose + soybean meal	--	--	PAA	--	123
--	soybean oil + lactose	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub> , CaCO <sub>3</sub>	PAA	--	124
CCM F-648	sucrose + lactose	--	--	PAA	-- <sup>2</sup>	125

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<sup>1</sup>PAA = Phenoxyacetic acid

<sup>2</sup>Penicillin G also produced by the addition of phenylacetic acid as the side chain precursor



The total synthesis of bisnorpenicillin V potassium salt was reported by Hoogmartens *et al.*<sup>149</sup>. The  $\alpha$ -isomer of t-butyl(3-carboxy-5-thiazolidinyl)phthalimido acetate was esterified, hydrazinolized, N-tritiated and cyclized to give a bisnorpenicillanate. This compound was then detritylated, acylated with phenoxyacetyl chloride and debenzylated by hydrogenolysis to afford potassium bisnorpenicillin V. The *in vitro* activity of this penicillin against penicillin-sensitive organisms was much lower than that of penicillin V.

Penicillins can also be synthetically modified to prepare numerous derivatives. The potassium salts of penicillins V and G can be treated with procaine HCl and N,N'-dibenzylethylenediamine diacetate to give the corresponding procaine and benzathine salts<sup>150</sup>, respectively. Mice *et al.*<sup>151</sup> recently prepared the sodium salt of penicillin V by treatment of the free acid with sodium 2-pyrrolidinone in methylene chloride and diethylamine. N, O-bis(trimethylsilyl) acetamide has been successfully used to N-silylate the 6-position of penicillin V<sup>152</sup>.

Mercury(II) acetate reacts with penicillins V and G to give corresponding acetoxymercury (II)salts. Stoodley<sup>153</sup> used this to synthesize acetoxyazetidinones where the thiazolidine ring has been ruptured and desulfurized. More recently, Wei and Weigle<sup>154,155</sup> have published and patented a synthesis of azetidinones via desulfurization of the sodium and potassium salts of penicillins V and G by Raney Nickel. Oxidation of the sulfur in penicillins V and G with H<sub>2</sub>O<sub>2</sub> in methylene chloride in the presence of formic acid afforded the corresponding sulfoxide in 85-100% yield<sup>156</sup>.

The kinetics of the reaction of amine and oxygen nucleophiles with a number of penicillins including penicillins V and G in aqueous solution at 35°C was studied by Bundgaard<sup>157</sup>. The results are suggested to serve as a reference system for evaluating chemical reactions involved in penicillin allergy e.g., reactions with nucleophilic groups of proteins in the body to yield immunogenic penicilloyl-protein conjugates.

The chemical reactivity of penicillins in the body, especially the stomach, is of major concern. Some pharmaceutical compounds form N-nitrosamines, many of which are known carcinogens, under simulated human stomach conditions. Ziebarth *et al.*<sup>158,159</sup> found that phenoxymethylpenicillin was indeed nitrosated in gastric juice at pH 2 and under simulated human stomach conditions. He also found that ascorbic acid inhibited nitrosation by at least 80%.

Kallmayer<sup>160</sup> synthesized nitrophenacyl esters of penicillins V and G from the precursor penicillins and found that the esters possessed much more chromatographic stability than the original penicillins. Polyethylene glycol has been used as a carrier polymer for the attachment, via end groups, of drugs such as penicillin V<sup>161</sup>.

## 5. Bioavailability and Pharmacokinetics

Literally hundreds of articles exist in the literature on the bioavailability and pharmacokinetics of  $\beta$ -lactam antibiotics. Specifically, reviews have been published on the bioavailability<sup>162-165</sup>, pharmacokinetics<sup>165-169</sup>, absorption<sup>170-171</sup>, protein binding<sup>172-175</sup> and toxicology<sup>172</sup> of  $\beta$ -lactam antibiotics including potassium penicillin V. The above list of reviews is not inclusive and is intended only as a representative sampling of the available literature. In addition, the ensuing discussion is not inclusive but only samples research done in the field.

Potassium penicillin V is rapidly absorbed into the blood stream of animals and humans with peak serum levels occurring within 0.75 hours. A large portion of potassium penicillin V is excreted in the urine as phenoxymethylpenicilloic acid, the major metabolite<sup>110-173</sup>. Table VIII gives a representative sampling of the literature available on the subject of bioavailability of potassium penicillin V.

The biological half-lives of penicillin V, G, oxacillin and propicillin<sup>183-184</sup> were found to be around 30 minutes while phenethicillin and ampicillin were 20 minutes and 1-2 hours, respectively. Comparison of the absorption rates of three salts of penicillin V i.e., calcium, potassium and benzathine showed that the potassium salt was more rapidly absorbed and had superior bioavailability<sup>185,186</sup>. Hedges *et al.*<sup>187</sup> found that effervescent formulations of phenoxymethylpenicillin allowed more rapid and consistent absorption than plain tablet preparations.

Several factors influence the gastrointestinal absorption of potassium penicillin V. Yoshimura and Kakeya<sup>188</sup> correlated acid stability of penicillins V and G with Taft's inductive substituent constants. They also found good correlation between lipophilicity, log P and the peak plasma level or relative bioavailability in mice and rats. Transfer mechanisms of potassium penicillin V in rat intestine *in vivo* have been studied by several groups<sup>189-191</sup>. Lipophilicity and pH were found to be extremely important in penicillin transport. Rollo<sup>189</sup> hypothesized that formation of penicillin V micelles might have a deleterious effect on absorption.

Radioactive labeling of potassium penicillin V with tritium or <sup>35</sup>S has aided researchers in delineating absorption mechanisms. Ryrfeldt<sup>192</sup> found that organic anions such as dinitrophenol, probenecid and azidocillin markedly decreased the radioactive uptake of tritiated penicillin V *in vivo* while cationic compounds had no effect. The pharmacokinetics of <sup>35</sup>S-phenoxymethylpenicillin in humans was studied by Hellstrom *et al.*<sup>193</sup>. Incomplete recovery of orally administered drug was due to poor absorption and decomposition before and after absorption. Thin Layer Chromatography (Section 6.52) and paper chromatography

Table VIII. Bioavailability of Potassium Phenoxymethylpenicillin

<u>Formulation</u>	<u>Vector</u>	<u>Dose</u>	<u>Elimination in urine (%)</u> <sup>1</sup>	<u>Peak Serum Level</u> <sup>2</sup>	<u>Ref.</u>
tablets, capsules	Human-adult	250 mg	--	3-4 mcg/ml	174
powder	Human-adult	38000 U/kg	--	8.4 mcg/ml <sup>3</sup>	175
granules	Human-adult	--	--	3.2 U/ml	176
tablets, syrup	Human-adult	10 <sup>6</sup> U	33-41, 19-26	9.3-11.6, 1.5-27. U/ml	177
tablets	Human-adult	2 x 10 <sup>5</sup> - 10 <sup>6</sup> U	35.7-43.1	--	178
bulk	Human-adult	0.4, 1 gram	37-43	6.1, 15 mcg/ml	179
oral	Human-baby	20000 U/kg	15	9.1 mcg/ml	180
oral	Human-adult	20000 U/kg	25.4	6.4 mcg/ml	180
oral	Human-adult	--	--	1.018 mcg/ml	181
I.V.	Rabbit	--	48.7	--	182

<sup>1</sup> Based on the major metabolite phenoxymethylpenicilloic acid

<sup>2</sup> All serum peaks occurred within 0.75 hours

<sup>3</sup> The calcium salt had a peak serum level of 8.52 mcg/ml

Section 6.51) after purification on Sephadex G10 was used as the analytical finish.

Quantitative structure-activity relation of penicillins V, G and 5 others with nucleophilic reactivity has been studied by Du and Liu using MO theory<sup>195</sup>. Penicillin V plus 10 other antibiotics were used by Garzia et al.<sup>195</sup> to apply a mathematical model to the study of quantitative structure-selectivity of antibiotics.

Subcutaneously implanted cotton threads followed by microbiological assay allowed Hoffstedt and Walder<sup>196</sup> to monitor the penetration of penicillin V into noninflammatory extravascular fluid in rabbits and human volunteers. A method for measurement of antibiotic levels i.e., penicillin V, in human interstitial fluid is described by Tan<sup>197-198</sup> and involves collection of small fluid samples via a small skin window technique.

Zero-order release of water soluble drugs such as penicillin V for topical application to the skin or the eye has been obtained by laminating the drug between thin films of polymers prepared from alkoxyethylacrylates and methacrylates<sup>199</sup>. To reduce skin irritation an outer coating of a non-irritating water swellable film was used.

The relation between the absorption of potassium penicillin V and the dissolution process was investigated by Stricker<sup>200</sup> in a Sartorius dissolution apparatus using simulated gastric fluid. The results were in good agreement with those obtained in analogous *in vivo* studies.

### 5.1 Enhancement and Inactivation of Penicillin V Activity

Many compounds have been found that inhibit the antimicrobial activity of penicillin V e.g., rat tissue homogenates<sup>201</sup>, animal serum<sup>202</sup>, guar gum<sup>203</sup> and vitamins (B<sub>1</sub>, B<sub>2</sub>, nicotanimide, p-aminobenzoic acid)<sup>204</sup>. Alternatively, compounds given concomitantly with penicillins can provide a synergistic effect. Cholesterol, cholesteryl acetate,  $\beta$ -sitosterol<sup>205-206</sup>, cholestyramine and sodium bicarbonate<sup>207</sup>, oxyphenbutazone<sup>208</sup>, chymotrypsin, chymopsin<sup>209</sup> and seven phenothiazine tranquilizers increased absorption and bioavailability of potassium penicillin V. Interestingly, penicillin G, administered orally, increased the blood serum levels of penicillin V and vice versa<sup>211</sup>. Interference in the assay for folic acid by *L. casei*<sup>212</sup> and the *L. leichanni* bioassay<sup>213</sup> of serum vitamin B<sub>12</sub> was noted for penicillin V.

### 5.2 Binding

The binding of many drugs including penicillin V by albumin and plasma protein has been reviewed by Vallner<sup>214</sup>. Chojnowski<sup>215-218</sup> and Saha and Roy<sup>219</sup> reported binding of penicillin V to human albumin and human and horse serum in the range of 50-70%. Among the reported analytical techniques that have been used to determine binding levels of penicillin V to

albumin (human or bovine ovine) or to lysozyme are fluorometry via the probe 1-anilinonaphthalene-8-sulfonic acid<sup>220</sup>, pulse radiolysis<sup>221-225</sup>, microcalorimetry<sup>226-227</sup>, electron spin resonance by spin-labeling with minoxyl radicals<sup>373</sup>, colorimetry via probes methyl orange<sup>228</sup> and 2-(4'-hydroxybenzeneazo)benzoic acid<sup>229</sup>, equilibrium dialysis<sup>230-231</sup> and inhibition of <sup>14</sup>C-labeled penicillin G<sup>233</sup>. Schiff *et al.*<sup>232</sup> found that DD-carboxypeptidase rapidly binds with penicillin V then decomposes to give phenoxymethylpenicilloic acid, the major metabolite.

### 5.3 Immunogenicity and Toxicity

Penicilloylation is purported to play a major role in the immunogenicity of penicillins. Immunogenicity of penicillins V and G has been studied by immunizing rabbits with penicillin before and after selective hydrolysis with 3,6-bis(dimethylamino-methyl)catechol<sup>234</sup>, hemagglutination inhibition<sup>235</sup>, kinetic analysis of the penicilloylation of imidazole groups of the histidine residue<sup>236-237</sup> and ultraviolet spectrophotometric analysis of the formation of DL-phenoxymethylpenicillenic acid from penicillin V<sup>238</sup>.

Penicillins are striking in their lack of toxicity. In fact, the estimated LD<sub>50</sub> of potassium phenoxymethylpenicillin determined by giving mice a 20% oral solution was found to be above 8000mg/kg or  $1.2 \times 10^6$  Units/kg<sup>66</sup>.

## 6. Methods of Analysis

The chemical and physical analysis of antibiotics has been extensively reviewed by Hughes *et al.*<sup>239-240</sup>. The following year Fairbrother<sup>241-242</sup> published reviews on the chemical and biological assays of antibiotics. These reviews present an excellent overview of literature pertaining to the assay of antibiotics prior to 1977.

### 6.1 Official Analytical Methods

For potassium penicillin V, the Code of Federal Regulations(CFR)<sup>243</sup> allows a choice of a microbiological agar diffusion assay, iodometric assay or hydroxylamine colorimetric assay to determine potency. The United States Pharmacopeia (USP)<sup>244</sup> uses an iodometric assay to determine potency while the British Pharmacopeia (BP)<sup>245</sup> and the European Pharmacopeia (EP)<sup>246</sup> list a potentiometric titration. In the BP and EP procedures, potassium penicillin V is dissolved in pH 4.6 acetate buffer and 1 N nitric acid is added. Using a mercury-mercury(I) sulfate reference electrode and a platinum indicating electrode, the penicillin is titrated with 0.02M Hg(II)NO<sub>3</sub>. The total degradation products are determined in similar fashion except no nitric acid is added to the penicillin solution and the titration is carried out immediately. Phenoxyacetic acid is determined by TLC

on silica gel G with chloroform: formic acid: methanol(80:15:5) as the mobile phase and detection by spraying with 0.15% potassium permanganate in 5% sulfuric acid.

In addition to the potency assay requirements, both the BP and the EP list identity tests for potassium penicillin V. Similarity of the infrared spectrum to that of standard material constitutes a positive identity test. Penicillin can also be reacted with penicillinase, iodine and then starch. If the solution turns blue the test is positive for penicillin.

## 6.2 Identity Tests

In addition to the identity tests listed in the official compendia, many other procedures exist in the literature for the identification of penicillins (Table IX).

## 6.3 Spectrophotometric Analysis

### 6.31 Ultraviolet Analysis

Lepidi and Nuti<sup>260</sup> reported a spectrophotometric method for the determination of ampicillin, methacillin, oxacillin, penicillin G and penicillin V where penicillinase was used to cleave the  $\beta$ -lactam ring prior to analysis. Addition of Cu (II) to the reaction mixture followed by UV at 255-280 nm allowed quantitation of the penicillin. Subsequent to alkaline hydrolysis penicillin V has been determined by reaction with diphenylpicrylhydrazyl radical<sup>261</sup> followed by UV analysis. Alkaline hydrolysis of the parent penicillins followed by addition of pH 8.0 barbiturate buffer and D(+) -glucosamine at 70°C for 2 hours allowed Shaikh *et al.*<sup>262</sup> to determine 6-aminopenicillanic acid in the presence of penicillins V, G or 6 other penicillins and their degradation products.

Phenoxymethylpenicillin in fermentation broth was assayed by acid decomposition followed by reaction with imidazole<sup>263,264</sup>. The penicillin content was corrected for p-hydroxyphenoxy-methylpenicillin content by subtracting the UV contribution at 306nm of an alkaline solution of the same primary solution. Zollner<sup>265,266</sup> used the measurement of the UV absorption at 320 nm along with IR spectroscopy to determine the stability of penicillin V in chloroform. The differential UV absorption spectra of various penicillins and their corresponding penicilloic acids provided Samuni<sup>88</sup> with a direct assay for  $\beta$ -lactamase activity.

### 6.32 Colorimetric Analysis

The reactivity of penicillins make them excellent candidates for colorimetric determinations. A compilation of colorimetric assays of penicillin V published after 1970 is shown in Table X. Penicillins V and G in biological fluids have been determined by (1) incubation with D-alanyl-D-alanine carboxypeptidase immobilized on poly(N,N-dimethylacrylamide) resin to give an

Table IX. Identity Tests for Penicillin V

<u>Type</u>	<u>Comment</u>	<u>Ref.</u>
Infrared	carbonyl absorption at 1790 $\text{cm}^{-1}$ monitored	247,248
Ultraviolet	Penicillins V, G + several others	249
Color Reactions	$\text{H}_2\text{SO}_4$ , $\text{H}_2\text{SO}_4$ -HCOH followed by TLC or IR	250
Color Reaction	$\text{H}_2\text{SO}_4$ -yellow; sodium chromatopate and $\text{H}_2\text{SO}_4$ -red violet; $\text{H}_2\text{SO}_4$ :HCOH-red brown	251,252
Color Reaction	Chromotropic acid + $\text{H}_2\text{SO}_4$ at $150^\circ\text{C}$	253
TLC	Penicillins V and G	254,255
TLC	Silica gel, $\text{CHCl}_3$ : $\text{Et}_2\text{O}$ :benzene:acetone:acetic acid (85:15:10:10:10); detection-spray with $\text{NH}_3$ , $\text{CuSO}_4$	256
TLC	Silica gel F <sub>254</sub> , butyl acetate:acetic acid: $\text{CH}_3\text{OH}$ :n-butanol:pH 7.3 phosphate buffer (80:4:5:15:20), detection - spray with 10% $\text{FeCl}_3$ : $\text{KFe}_3(\text{CN})_6$ :HCl (1:2:6)	257
TLC	Benzene:ethyl acetate:formic acid (80:15:6), $R_f = 0.28$	258
TLC	Carboxamide derivative prepared, silica gel GF <sub>254</sub> with ethyl acetate, detection - spray with $\text{NaNO}_2$ - $\text{H}_2\text{SO}_4$ ( $R_f \sim 0.7$ )	259

inactive and irreversible enzyme-antibiotic complex, (2) washing the complex, (3) incubation of the washed complex with a substrate capable of being hydrolyzed to D-alanine by residual, uncomplexed enzyme and (4) colorimetric determination of the D-alanine formed by a coupled enzyme reaction involving D-amino acid oxidase plus peroxidase, the final measurement being the change in absorption of o-anisidine at 460 nm<sup>367</sup>.

## 6.4 Titrimetric Analysis

### 6.41 Coulometry

Mndzhoyan *et al.*<sup>282</sup> determined several penicillins including penicillin V by alkaline hydrolysis followed by titration with bromine. The assay precision was found to be comparable to the conventional iodometric assay. An absolute coulometric method based on the titration of hydrolyzed penicillins (1M NaOH for 5-10 minutes) with coulometrically generated Mercury (II) is described by Forsman<sup>283</sup>. An amalgamated gold plate is used as the anode and the titration is performed in a pH 4.6 acetate buffer. Penicillins V and G, ampicillin, cloxacillin, 6-amino-penicillanic acid, penicillamine and penicilloate were determined by this method.

### 6.42 Iodometry

Iodometric methods for the assay of penicillins were first described by Alicino<sup>284</sup> in 1946. Since 1970 numerous variations in this method have been published. The compendial methods require the standard and sample to be dissolved in pH 6.0 phosphate buffer followed by addition of 1.0N NaOH (15 minutes reaction time), 1.2N HCl, excess 0.01N iodine (15 minutes) and finally titration with 0.01N sodium thiosulfate.

Moehrle and Luther<sup>285-286</sup> reported a periodometric assay used for the determination of penicillins such as penicillins V and G. The penicillins were hydrolyzed in alkaline media, neutralized and subjected to peroxide oxidation in an acetate buffer solution. These solutions were then treated with aqueous sodium metaperiodate and sodium arsenite and the excess arsenite back titrated with an iodine solution.

Ibrahim *et al.*<sup>287,288</sup> used iodine monochloride for the iodometric determination of penicillins. This method has been applied successfully to penicillins V and G and can also be used for determining penicillin in its various dosage forms. Nayak *et al.*<sup>289</sup> also determined five penicillins including penicillin V by addition of iodine monochloride with the liberated iodine being titrated with chloramine T. The stoichiometry of penicillin: chloramine T was 1:4. No interference from excipients was found.

Alicino<sup>290</sup> has developed a bulk or batching assay for the determination of penicillin V, G and 5 other penicillins by



Table X. Colorimetric Analysis of Penivillin V

<u>Penicillin</u>	<u>Reagents</u>	<u>Solvent</u>	<u>Reaction Conditions</u>	<u><math>\lambda</math> (nm)</u>	<u>Limit of Detection (mcg/ml)</u>	<u>Ref.</u>
V	1.2M Imidazole + $10^{-3}$ M HgCl <sub>2</sub>	pH 6.8 phosphate buffer	60°C	325	0.5	267
V,G + 4 others	1M ammonium vanadate + conc. H <sub>2</sub> SO <sub>4</sub>	Water	10 minutes at 100°C	750	100	268
NaPen G + V	methylene blue + pH 6.8 phosphate buffer, extract with CHCl <sub>3</sub>	CHCl <sub>3</sub>	--	640	--	269
V, G + 11 others	alkali + acid + dithionitrobenzoic acid (Ellman's Reagent)	Water	--	412	--	270
V, G	Lowry Reagent <sup>1</sup>	Water	15 minutes	500	500	271
V,G	acid hydrolysis + Cr (VI) at pH 3.4 + metol <sup>2</sup>	Water	30 minutes	--	40	272, 273

Table X.,continued:

<u>Penicillin</u>	<u>Reagents</u>	<u>Solvent</u>	<u>Reaction Conditions</u>	<u><math>\lambda</math> (nm)</u>	<u>Limit of Detection (mcg/ml)</u>	<u>Ref.</u>
V,G	azure B + extract into $\text{CHCl}_3$	$\text{CHCl}_3$	RT <sup>3</sup>	634	--	274
V,G	formaldehyde + $\text{H}_2\text{SO}_4$	Water	100°C	450	30	253
V	hydroxylamine + $\text{Fe}^{+3}$	pH 7.0 acetate	RT	490	--	275- 278
V,G	penicillinase + $\text{HgCl}_2$ + molybdoarsenic acid	Water	RT	650	1.0	279-281

---

1 Phosphomolybdic acid-tungstic acid mixture +  $\text{CuSO}_4$

2 4-methylaminophenol:sulfate (2:1)

3 Room Temperature

iodometry. The penicillins are reacted for 1-2 hours with 0.02N N-bromosuccinimide, then acetic acid and potassium iodide are added and finally the liberated iodine is titrated with sodium thiosulfate. This method was not useful for determining stability. In a similar fashion, 3-bromo-4, 4-dimethyl-2-oxazolidinone<sup>291</sup> has been used in place of N-bromosuccinimide for the quantitative determination of penicillins.

#### 6.43 Miscellaneous

Blazsek-Bodo *et al.*<sup>292,293</sup> hydrolyzed penicillins to penicillamines with NaOH then titrated the resultant solution potentiometrically with  $5 \times 10^{-3}$  M Hg ( $\text{ClO}_4$ )<sub>2</sub>. The method is based on the complexation of  $\text{Hg}^{2+}$  with penicillamine and is sensitive to about 1 mg of penicillin V.

Potassium penicillin V, penicillin G and 6-aminopenicillanic acid have been determined by potentiometric-argentometric methods<sup>294,295</sup>. After alkaline hydrolysis and addition of acetic acid, the resulting solutions were titrated with  $\text{AgNO}_3$ . The mechanism of the reaction and the complexation of  $\text{Ag}^+$  with the hydrolysis products were discussed.

Micro and semimicro quantities of potassium penicillins V and G in tablets were determined directly by using a standard solution of  $\text{Fe}(\text{NO}_3)_3$  as the titrant and 1% alcoholic phenol as the indicator<sup>296</sup>. The method can be used satisfactorily for tablets at the end of their expiration date when penicilloic acid may be present in significant amounts.

### 6.5 Chromatographic Analysis

#### 6.51 Paper Chromatography

Hellstrom *et al.*<sup>193</sup> described a paper chromatographic procedure using n-butanol:acetic acid:water (40:10:50) v/v (descending) as the solvent for the separation and determination of penicillin V and its metabolic products. The  $R_f$  values for penicillin V, penicillin V penicilloic acid, p-hydroxyphenicillin V, 6-aminopenicillanic acid and penicillin V penicic acid are 0.91, 0.80, 0.86, 0.35 and 0.29, respectively.

#### 6.52 Thin Layer Chromatography

Recently, Hendrickx<sup>298</sup> published an article on the Thin Layer Chromatography (TLC) of 18 penicillins including penicillin V on silica gel and silanized silica gel using 35 different mobile phases. Iodine vapor was used as the mode of detection. A compilation of the TLC separation and quantitation of penicillin V, other penicillins and degradation products published after 1970 is shown in Table XI.

#### 6.521 High Performance Thin Layer Chromatography

High Performance Thin Layer Chromatography (HPTLC) was used by Kruezig<sup>315,316</sup> to analyze penicillin V fermentation broth.

Lactose, sucrose, soybean oil, phenoxyacetic acid, penicillin V ( $R_f = 0.44$ ) and p-hydroxyphenicillin V ( $R_f = 0.34$ ) were determined and quantitated with a single chromatographic run.

Derivatization after chromatography was performed with an automatic spraying device, the measurement perpendicular to the direction of the chromatogram. The HPTLC plates were silica gel 60F<sub>254</sub> (20 x 10 cm) with the eluent being toluene:ethyl acetate:acetic acid (40:40:20). The detection spray was composed of a mixture of 2 mL aniline, 2 grams diphenylamine, 10 mL phosphoric acid and 88 mL methanol.

The separation and quantitation of 6 penicillins from their respective penicilloic and penilloic acids by HPTLC has been accomplished by Aboul Khier *et al.*<sup>317</sup>. A solvent system of methanol: 0.01M KH<sub>2</sub>PO<sub>4</sub>: acetonitrile (8:4:1), pH adjusted to 4.1 was used on plates coated with RP<sub>18</sub>. The detection was reflectance mode densitometry at 230 nm. The  $R_f$  values for penicillin V, penicillin V penicilloic acid and penicillin V penilloic acid were 0.46, 0.67 and 0.75, respectively. The system also works for penicillin G, propicillin, fluoxacillin, oxacillin and ampicillin.

#### 6.522 Reversed Phase Thin Layer Chromatography

The lipophilic character of drugs such as penicillins can be expressed by the chromatographic  $R_M$  value, determined by TLC and defined by the equation  $R_M = \log (1/R_f - 1)$ . Table XII summarizes research published on this topic since 1970.

#### 6.53 High Performance Liquid Chromatography

A compilation of HPLC procedures for the determination and quantitation of penicillin V, its potassium salt, degradation products and metabolites is given in Table XIII.

##### 6.531 Ion Liquid Chromatography

An ion chromatographic procedure developed by Whitaker *et al.*<sup>347</sup> was able to determine sodium or potassium in their respective phenoxyethylpenicillin salts in 9-16 minutes with a relative precision of  $\pm 2.5\%$ .

##### 6.532 Liquid Chromatography-Mass Spectrometry

Recently, McDowall *et al.*<sup>24</sup> reported a combined microbore LC-MS procedure for the determination of penicillin G and methicillin. Using a Whatman 250 x 1mm Partisil 10 ODS-3 column and gradient elution going from 25% methanol to 50% methanol in ammonium carbonate over 15 minutes at a flow rate of 30 microliters min<sup>-1</sup>, they were successful in separating and determining the two penicillins. The mass spectrometric mode of detection was positive chemical ionization with ammonia. Penicillin V should be amenable to this type of analysis.

#### 6.54 Gas Chromatography

Hishta *et al.*<sup>348</sup> reported that the relative retention times for the gas chromatographic separation of silylated penicillin G, D-

Table XI. Thin Layer Chromatographic Systems for the Detection and Determination of Penicillin V

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>R<sub>f</sub></u>	<u>Comment</u>	<u>Ref.</u>
acetone:acetic acid (95:5)	silica gel	<sup>35</sup> S-penicillin V	--	0.64 penicilloic acid (0.20) p-OH pen V (0.63) 6-APA (0.35) penicic acid (0.10)	--	193
ethyl acetate:acetic acid:H <sub>2</sub> O (80:10:10)	--	--	--	0.90	--	193
isoamyl acetate:methanol:formic acid:H <sub>2</sub> O (65:20:5:10)	--	--	--	0.47	--	193
pH6.0 phthalate buffer:acetone (80:20)	silanized silica gel HF <sub>254</sub>	spray with starch then iodine - NaN <sub>3</sub>	--	0.39	--	300
acetic acid:n-butyl acetate:0.1M phosphate buffer:n-butanol:ethanol (20:40:12:7.5:7.5)	Silica gel 60 F <sub>254</sub>	spray with reagent composed of KI, chloroplatinic acid and HCl	--	0.62	A	301

Table XI. (continued):

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>R<sub>f</sub></u>	<u>Comments</u>	<u>Ref.</u>
n-butanol: water:ethanol: acetic acid (5:2:1.5:1.5)	Silica gel G	spray with 2N NaOH then I <sub>2</sub> -NaN <sub>3</sub> followed by 1% starch	--	0.84	B	302
n-butanol:water:acetic acid (4:1:1)	--	--	--	0.78	--	302
acetone: acetic acid (95:5)	--	--	--	0.66	--	302
85% aqueous acetone	--	--	--	0.70	--	302
n-butanol:acetic acid:H <sub>2</sub> O (60:15:25)	silica gel G	spray with 0.2% PtCl <sub>4</sub> :20% KI:4% HCl:acetone (1:0.1:0.1:20)	0.05 mcg	0.82	C	303
acetone:methanol (1:1) or isopropanol:methanol (1:1)	silica gel	spray with 10% FeCl <sub>3</sub> :5% K <sub>3</sub> Fe(CN) <sub>6</sub> :16% H <sub>2</sub> SO <sub>4</sub> (2:1:7)	0.2 mcg	--	D	304
ethyl acetate:chloroform: formic acid (70:30:4)	silica gel	UV at 254 nm or spray with 0.5% alcoholic Rhodamine B	1.0 mcg	--	E	305

Table XI. (continued):

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>R<sub>f</sub></u>	<u>Comments</u>	<u>Ref.</u>
n-heptane:acetic acid (95:5)	silica gel GSK	--	--	--	--	306
n-propanol:28% aqueous ammonia (3:1)	silufol	--	--	--	--	307
water:acetic acid: isopropanol:CH <sub>3</sub> OH (50:16.5:7.1:5)	Polyamide	Spray with 0.5% bromine or 0.25% fluorescein Na	3.0 mcg	--	F	308
acetone:acetic acid:H <sub>2</sub> O (0.5:5:3.2)	silica gel	--	--	--	G	309
acetone:acetic acid:H <sub>2</sub> O (95:5:3.2) or acetone:acetic acid (95:5) followed by methanol:n-butanol: formamide:heptane (46:16:11:5)	silica gel	Spray with 0.01N iodine containing NaN <sub>3</sub> , then 1% starch	1-3 mcg	1.00 penillic acid (0.25) penicilloic (0.33) penilloic (0.73) penicillenic (1.15)	H	310- 312

Table XI (continued):

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>R<sub>f</sub></u>	<u>Comments</u>	<u>Ref.</u>
acetone:acetic acid (19:1)	silica gel	Spray with starch- iodine	--	--	I	313
ethyl acetate:chloroform: acetone (9:1:0.5)	silica gel	Spray with 1% FeCl <sub>3</sub> :1% K <sub>3</sub> Fe(CN) <sub>6</sub> (1:1)	--	--	J	314

- 
- A Method is applicable to drug raw material and liquid formulations  
 B Separates penicillins V and G from their respective degradation products  
 C Method can be used to determine several common penicillins and their metabolites  
 D Method can detect ampicillin, penicillin G and several other penicillins  
 E Method is used for the determination of phenoxyacetic acid in potassium penicillin V  
 F Useful in the separation of carbenicillin, methicillin, dicloxacillin, oxacillin, penicillin V and G  
 G Semiquantitative determination of an admixture of penicillin V  
 H Also applicable for the control of penicillin V production, isolation from fermentation broth and purification  
 I Determination of penicilloic acid in the presence of penicillin V  
 J Determination of p-hydroxyphenoxyacetic acid in penicillin V fermentation samples



Table XII. Reversed-Phase Thin Layer Chromatography of Penicillin V

<u>Stationary Phase</u>	<u>Mobile Phase</u>	<u>R<sub>M</sub></u>	<u>Comment</u>	<u>Ref.</u>
isobutyl alcohol	water	--	R <sub>M</sub> correlated with Hansch $\pi$ value	318,320
silicone oil	sodium acetate-veronal buffer	1.17 (pH 2.6) 0.89 (pH 9.4)	Pen V less ionized and more lipophilic at lower pH	321
silicone DC 200	0.05M phosphate pH 7.4 - acetone	--	antibacterial activity correlated with lipophilicity	322
n-octanol	water	0.34 (pH 3) -0.37 (pH 4) -0.87 (pH 5)	linear correlation between R <sub>M</sub> and log partition coefficient	323
water-saturated alkanols	aqueous buffers	--	separates pen V and G	324

Table XIII. HPLC Separation Systems for Penicillin V

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Retention Time (minutes)</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>Comment</u>	<u>Ref.</u>
Anion Exchange resin-1 meter	0.02M NaNO <sub>3</sub> in 0.01M pH 9.15 borate buffer	0.45	15.0	UV at 254 nm	20 ng	A	325
Styrene-divinyl- benzene 40-70 micrometers 2.5 x 600 mm	phosphate buffer:methanol	0.50	various	UV at 250 nm	--	B	326
Lichrosorb RP-8	phosphate buffer:methanol	--	various	UV at 220 nm	--	C	328
Octadecylsilane	phosphate buffer pH 2.5 to 7.5:methanol	--	various	UV	--	D	329
RP-8 10 micrometers 250 x 4.6 mm I.D.	methanol:pH 3.5 phosphate buffer (53:47)	1.0	12.3 p-OH penicillin V-4.9 phenoxyacetic acid-5.6 penicilloic acid - 6.4 penilloic acid - 7.2 penicillenic acid - 18.2	UV at 254 nm	--	--	330

Table XIII. (continued):

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Retention Time (minutes)</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>Comment</u>	<u>Ref.</u>
Nucleosil RP-8 5 micrometers 250 x 4.6 mm I.D.	acetonitrile:0.01M KH <sub>2</sub> PO <sub>4</sub> pH adjusted to 4.1 (20:80)	1.5	13.1 penilloic acid- 3.0 penicilloic acid - 9.0	UV at 254 nm	--	E	331
μ-Bondapak 10 micrometers 300 x 4.6 mm I.D.	NH <sub>4</sub> OAc:CH <sub>3</sub> CN:THF (82:10:8)	1.0	--	UV at 254 nm	--	F	332
C-18 10 micrometers	CH <sub>3</sub> CN:pH 6.0 phosphate buffer (28:72)	1.0	2.2	UV at 271 nm	--	G	100
C-18	--	--	--	Convert to 6-APA post column- reaction with OPA- fluorometry	1 ng	--	333

Table XIII. (continued):

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Retention Time (minutes)</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>Comment</u>	<u>Ref.</u>
Spherisorb C-18 5 micrometers	CH <sub>3</sub> CN:0.01M NaH <sub>2</sub> PO <sub>4</sub> , 0.01M EDTA pH 6.5 16.5% CH <sub>3</sub> CN → 31.5% in 16 minutes	2.0	14.2 penicillin G - 12.5	precolum - imidazole/HgCl <sub>2</sub> UV at 325 nm	1 mcg/mL	H	334
RP-8 10 micrometers 250 x 4.6 mm I.D.	CH <sub>3</sub> OH:pH 3.3 phosphate buffer (52:48)	1.5	7.2 p-OH phenoxyacetic acid - 3.0 phenoxyacetic acid - 3.2 penicilloic acid - 3.6 penilloic acid - 4.6 penicillenic acid - 11.0 penicillin G - 5.2	UV at 254 nm	--	I	335
Partisil 10 micrometers	CH <sub>3</sub> CN:water 5% CH <sub>3</sub> CN → 76% in 20 minutes	1.3	penicillin V phenacyl ester - 12.0	Precolumn -α,p- dibromoacetone UV at 254 nm	--	--	336

Table XIII. (continued):

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Retention Time (minutes)</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>Comment</u>	<u>Ref.</u>
Lichrosorb RP-8 10 micrometers 250 x 3.2 mm I.D. 50°C	CH <sub>3</sub> OH:pH 7.0 phosphate buffer (60:40)	1.5	3.75	UV at 220 nm	--	J	337
C-18	CH <sub>3</sub> CN:0.01M H <sub>3</sub> PO <sub>4</sub> 20% CH <sub>3</sub> CN→40% in 20 minutes	1.0	18	UV at 220 nm	5 PPB	K	338
C-18 5 micrometers 150 x 4.6 mm I.D.	CH <sub>3</sub> OH:0.01M NaH <sub>2</sub> PO <sub>4</sub> (35:65)	1.0	18	UV at 225 nm	--	L	339
microbondapak- NH <sub>2</sub>	Acetic acid:CH <sub>3</sub> OH:CH <sub>3</sub> CN: H <sub>2</sub> O (2:4:7.5:86.5)	1.5	12.5 p-OH penicillin V- 3.5	UV at 254 nm	--	M	340
C-18 10 micrometers	CH <sub>3</sub> OH:0.01M NaH <sub>2</sub> PO <sub>4</sub> (35:65)	1.0	22	UV at 225 nm	--	--	341

Table XIII. (continued):

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Retention Time (minutes)</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>Comment</u>	<u>Ref.</u>
C-18 50 micrometers	CH <sub>3</sub> OH:0.05M NH <sub>4</sub> CO <sub>3</sub> (30:70)	0.5	~25	UV at 254 nm	--	--	342
Chromegabond C-18 10 micrometers	CH <sub>3</sub> CN:CH <sub>3</sub> OH: pH 4.7 0.01M KH <sub>2</sub> PO <sub>4</sub> (19:11:70)	1.0	8.4	UV at 225 nm	1 mcg/mL	N	343
Ultrasphere C-18 5 micrometers	CH <sub>3</sub> CN:pH 3.0 (NH <sub>4</sub> ) <sub>3</sub> PO <sub>4</sub> (30:70)	1.0	12.0 p-OH penicillin V - 6.0 phenoxyacetic acid - 3.8 p-OH phenoxy- acetic acid - 2.5	Electrochemical in series with UV (254 nm)	--	M	344
Spherisorb C-18 5 micrometers	CH <sub>3</sub> CN:0.01M pH 6.5 phosphate buffer, 0.01M EDTA (20:80)	1.5	12.0 penicillin G - 10.0	Precolumn- Imidazole/HgCl <sub>2</sub> , UV at 254 nm	--	--	345

Table XIII. (continued):

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Retention Time (minutes)</u>	<u>Detection</u>	<u>Limit of Detection</u>	<u>Comment</u>	<u>Ref.</u>
C-18	CH <sub>3</sub> CN:0.01M pH 6.5 KOAc (20:80)	1.6	3.1 Chloramphenicol - 5.1	UV at 215 nm	30 mcg/mL	N	346

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**Comments to Table XIII**

- A = Penicillenic and penicilloic acids were also separated and quantitated.  
 B = Separates penicillin V from other penicillins  
 C = Method compared to nonchromatographic pharmacopoeial methods  
 D = Determination of capacity factors of penicillins V and G  
 E = Separates penicillin G, ampicillin, oxacillin, propicillin, amoxicillin, cloxacillin and fluoxacillin from closely related degradants  
 F = Penicillin V was purified on analytical, preparative and process scales  
 G = Determined in syrups  
 H = Determination of penicillins V, G, K, N & X in fermentation media  
 I = 1,3,5-trimethoxybenzene used as internal standard  
 J = Separates phenoxyacetic acid and 6-aminophenylacetic acid and is applicable to the monitoring of fermentation and recovery  
 K = Penicillins were extracted from milk with acetonitrile then partitioned between buffer and organic solvents  
 L = Separates penicillins V and G, ampicillin, methacillin and oxacillin  
 M = Analysis in fermentation borth  
 N = Applicable to the quantitative analysis of dosage forms

phenethicillin, L-phenethicillin and penicillin V were 1.65, 1.60, 1.71 and 2.05 (relative to 5- $\alpha$ -cholestane = 1.00) respectively, at 245°C on a column containing 2% OV-17 on 80-100 mesh Supelcoport. The relative retention times for silylated methicillin, oxacillin, cloxacillin and dicloxacillin were 1.51, 1.58, 2.16 and 2.83, (relative to 5- $\alpha$ -cholestane-3-one) respectively at 275°C on the same column.

A pyrolysis gas-liquid chromatographic procedure for the direct characterization and differentiation of 14 penicillins and cephalosporins including penicillin V was published by Roy and Szinai<sup>349</sup>. The chromatographic conditions employed were flame ionization detection with a carrier gas flow of 60ml/min on a column containing XE-60 on 80-100 mesh Gas Chrom Q. The column temperature was set at 100°C. Excellent linearity was obtained in the 10 ng to 100 mcg range with the limit of detection found to be 0.5 ng.

#### 6.6 Electrophoresis

Smither and Vaughan<sup>350</sup> examined 50 antibacterial agents including potassium penicillin V by a high voltage electrophoretic technique which determined their migration distances in 1% Bacto-agar and 1% agarose gels with pH 6.0 and 8.0 buffer systems consisting of tris and succinic acids. The field strength was 20 V/cm. Comparison of the different migration distances in the 2 gels formed the basis for identification. Bioautography, using *Bacillus cereus* var. *mycoides* or *Micrococcus luteus*, was used to visualize the antibiotics. The method was satisfactory for identifying many antibiotics in animal tissues, animal feeding stuffs, urine and pharmaceutical preparations.

#### 6.7 Polarography

A compilation of published methods for the determination of penicillin V by polarographic techniques is shown in Table XIV.

#### 6.8 Microbiology

Pertinent data for the performance of 83 different microbiological assays for antibiotics have been presented by Arret *et al.*<sup>358</sup>. Included are official methods for all antibiotics approved for human use in the United States plus methods for 10 other antibiotics. Table XV gives a representative sampling of the results published since 1971.

#### 6.9 Miscellaneous Analyses

##### 6.91 Fluorometry

Penicillin V and penicillin V penicilloic acid were determined by Tsuji *et al.*<sup>363</sup> via a fluorometric method based on the formation of a fluorescent Schiff base. This fluorphore is formed by reaction of 5-(dimethylamino)naphthalene-1-sulfonylhydrazine with penicillin V penicilloaldehyde generated



Table XIV. Polarographic Methods for the Determination of Penicillin V

<u>Analyte Preparation</u>	<u>Electrolyte</u>	<u>Experimental Conditions</u>	<u>Comments</u>	<u>Ref.</u>
Aqueous solution	1N NaOH	SCE, 4% agar-KCl salt bridge, DME	peak at 0.65V linear: 1-50 mcg/mL	351
acidic hydrolysis at 90°C	pH 4 buffer	SCE, DME	0.01-1.0 mg/mL tablets, syrup	352 -355
alkaline hydrolysis	pH 4.6 buffer with excess Cu <sup>2+</sup>	Stripping, SCE, hanging Hg drop electrode	peak at -0.10V, 20 ng/ml detectable	356
aqueous	pH 9.2 borate buffer with 0.05% Triton X-100	Flow injection, DME, SCE, constant potential of + 0.04V vs SCE	Determination of penicilloic acid content	357

Table XV. Microbiological Methods of Analysis of Penicillin V

<u>Microorganism</u>	<u>Experimental Conditions</u>	<u>Comments</u>	<u>Ref.</u>
Various	turbidimetry	holding coil - 1 hour, automated	359
<i>Bacillus mycoides</i>	paper disc dipped in secretions	limit - 0.15 units/mL	360
<i>Bacillus subtilis</i> , <i>Sarcina lutea</i> , <i>Pseudomonas aeruginosa</i>	agar-diffusion plate	blood and serum	361
Various	filter paper disc plated on thin-layer agar plates	limit - 5 pmoles/mL, inactivates R39 DD-carboxypeptidase	362

by the reaction of penicilloic acid with  $\text{HgCl}_2$  in an acidic medium. Assay of urine by this method after IV injection of penicillin V in rats gave similar results to a microbiological assay and the colorimetric imidazole method. About 9-15% of the dose was excreted as penicilloic acid after 3 hours.

#### 6.92 Penicillin Sensitive Enzyme Electrode

An electrode coated with acrylamide gel containing penicillinase was used by Papariello *et al.*<sup>364,365</sup> to determine penicillins V and G in pharmaceutical dosage forms in the concentration range of  $10^{-4}$  to  $5 \times 10^{-2}\text{M}$ . The enzyme hydrolyzes penicillins and the  $\text{H}^+$  produced is determined potentiometrically. Penicillin V has been determined in fermentation broth<sup>366</sup> with this type of electrode using a continuous-flow system.

The enzyme penicillinase immobilized on glass beads has been made part of a single bead string reactor used in a flow injection sample processing system for the determination of penicillins including penicillins V and G<sup>368</sup>. Penicillins V and G in tablets, injectables and fermentation broth were selectively determined in the 0.05 to 0.50 mM range and at a frequency of 150 injections per hour.

#### 6.93 Enzyme Thermistry-Flow Injection Analysis (ET-FIA)

Decristoforo and Danielsson<sup>369</sup> described a new, automated flow injection analytical method for the rapid and specific determination of  $\beta$ -lactam antibiotics including potassium penicillin V. The enthalpies of enzymic reactions were measured in continuous flowing streams by means of thermistors. Enzyme reactions were carried out on small solid bed reactor columns, the selectivity for various analytes being exclusively dependent on the chosen enzyme system. Statistically, no significant difference was found when the ET-FIA method was compared to published HPLC methods. The minimum amount of potassium penicillin V detectable by ET-FIA is 1 microgram. Sample capacity is 40-45 samples per hour and the time of analysis for one sample about 80 seconds.

#### 6.94 Enzymic Enthalpimetry

An enzymic enthalpimetric procedure for the determination of sodium penicillin G, sodium ampicillin and potassium penicillin V in pure and dosage forms is described by Grime and Tan<sup>370</sup>. Two concurrent reactions i.e., penicillin reacting with penicillinase in water to afford the penicilloic acid and  $\text{TrisNH}_2$  plus  $\text{H}_3\text{O}^+$  to give  $\text{TrisNH}_3^+$  plus water are used to define the parameters of the procedure. For example,  $\Delta\text{H}$  for potassium penicillin V was experimentally determined to be  $120 \pm 1.3 \text{ kJ mol}^{-1}$ .

### 6.95 Use as an Analytical Reagent

The potassium salts of penicillins V and G have been used as analytical reagents in the determination of Cu(II) and Fe(III). Tiwari and Chakrawarti<sup>371,372</sup> added a 2% solution of potassium penicillin V or G to  $\text{Fe}(\text{NO}_3)_3$  at pH 3-5.5. The resulting precipitate was weighed after drying and the Fe(III) subsequently quantitated. Fe(III) was also determined by titration with 0.02M potassium penicillin V or G using a 1% ethanol solution of phenol as indicator at pH 3.5-4.0. The formation of olive-green complexes with Cu(II) and red-brown complexes with Fe(III) allowed the same researchers to use potassium penicillin V and G for spot test detection<sup>297</sup> and photometric determination of Cu(II) and extraction-photometric determination of Fe(III).

Aqueous or methanolic solutions of copper acetate or sulfate, cobaltous nitrate, ferric chloride and ceric sulfate developed color when sprayed on TLC plates impregnated with penicillin V<sup>299</sup>.

## 7. Determination in Body Fluids and Tissues

Penicillin V has been determined in biological fluids and tissues by several different methods of analysis in combination with various extraction and purification techniques. Table XVI presents a representative sampling of the results that have been published since 1970. As can be seen several types of analytical procedures have been used to determine penicillin V in biological fluids.

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Table XVI. Determination of Phenoxymethylpenicillin In Biological Fluids and Tissues

<u>Analytical Method</u>	<u>Extraction-Purification</u>	<u>Biological System</u>	<u>Internal Standard</u>	<u>Ref.</u>
HPLC with post column derivatization	Protein precipitation	Plasma, whole blood, urine, lymph	--	327
TLC	--	human milk	--	304
TLC	Sephadex G10	urine	<sup>35</sup> S-penicillin V	193
Colorimetric	Acidification followed by extraction with chloroform	urine	--	270
Microbiological	--	serum, urine	--	361
Microbiological	--	oral, pharyngeal mucosal secretions	--	360
Polarographic	Ultrafiltration	serum	--	351
Microbiological	--	human serum, cow's milk	--	362
Microbiological-Colorimetric	Incubation with enzyme immobilized on acrylamide	serum, urine	--	367
Fluorometric	--	urine	--	363
HPLC	Extraction with ethyl ether after acidification	serum	chloramphenicol	346

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## *Analytical Profile of Primidone*

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## 1 Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

5-Ethylidihydro-5-phenyl-4,6[1H, 5H]-pyrimidinedione,  
5-Ethylperhydro-5-phenylpyrimidine-4,6-dione,  
5-Ethyl-5-phenylhexahydropyrimidine-4,6-dione,  
5-Phenyl-5-ethylhexahydropyrimidine-4,6-dione,  
2-Deoxyphenobarbital.

#### 1.1.2 Generic Names

Primidon, Primidonum, Hexamidinum, Primaclone,  
Desoxyphenobarbital.

#### 1.1.3 Trade Names

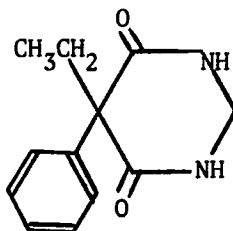
Cyral, Granmid, Lepsiral, Liskantin, Majsolin,  
Medi-Pets, Midone, Mylepsine, Mylepsinium, Mysedone,  
Mysoline, Neurosyn, Primoline, Prysoline, Resimatil,  
Sertan.

### 1.2 Formulae

#### 1.2.1 Empirical

$C_{12}H_{14}N_2O_2$ .

#### 1.2.2 Structural



#### 1.2.3 CAS Registry No.

[125-33-8]

#### 1.2.4 Wiswesser Line Notation

T6MV DVMTJ C 2 CR



### 1.3 Molecular Weight

218.3

### 1.4 Elemental Composition

C 66.03%, H 6.47%, N 12.84%, O 14.66%.

### 1.5 Appearance, Color, Odor and Taste

Primidone occurs as white odorless crystalline powder with a slightly bitter taste.

## 2 Physical Properties

### 2.1 Melting Range

279 to 284°C (1).

### 2.2 Solubility

Soluble 1 in 2000 of water and 1 in 170 of alcohol, practically insoluble in most other organic solvents (1).

### 2.3 Stability

Commercially available primidone tablets and oral suspension have expiration dates of 5 years following the date of manufacture.

### 2.4 Crystal Structure

Anticonvulsant agents effective in the treatment of grand mal epilepsy are characterized by having at least one phenyl group on the asymmetric carbon. The suggestion that the configuration of the substituents at this position may affect the anticonvulsant activity (2-4) lead Yeates and Palmer (5) to determine the crystal structure of primidone by direct methods and refined by full-matrix least-square calculations.

The crystal data were presented in Table 1. The numbering scheme is shown in Fig. 1. A view of the molecule along C(2)-C(5) is represented in Fig. 2. Table 2 shows the atomic parameters. No significant deviations from the expected values are observed for bond lengths and angles (Table 3) but C(13)-C(14) and C(14)-C(15) showed somewhat low values. Table 4 lists the torsion angles. A boat

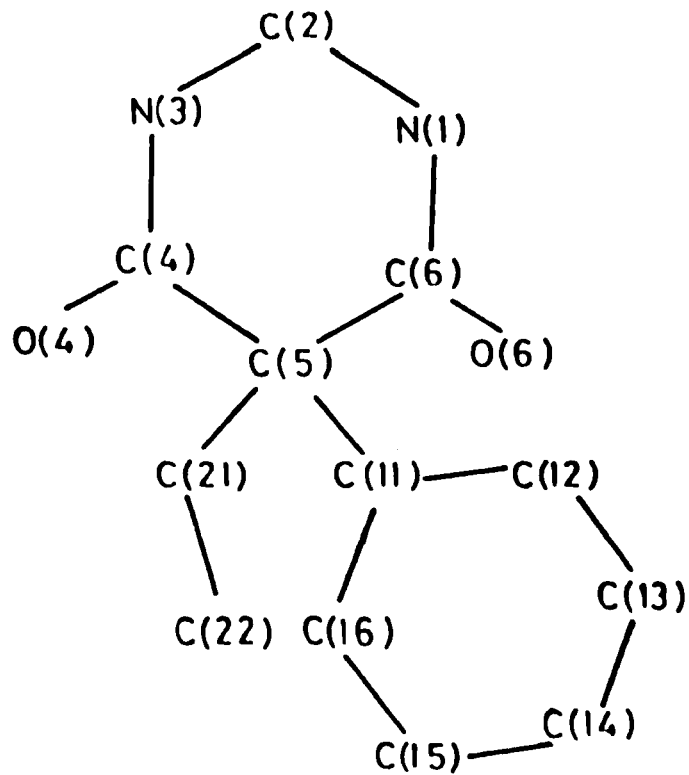


FIGURE 1: ATOMIC NUMBERING OF PRIMIDONE MOLECULE ( 5 )

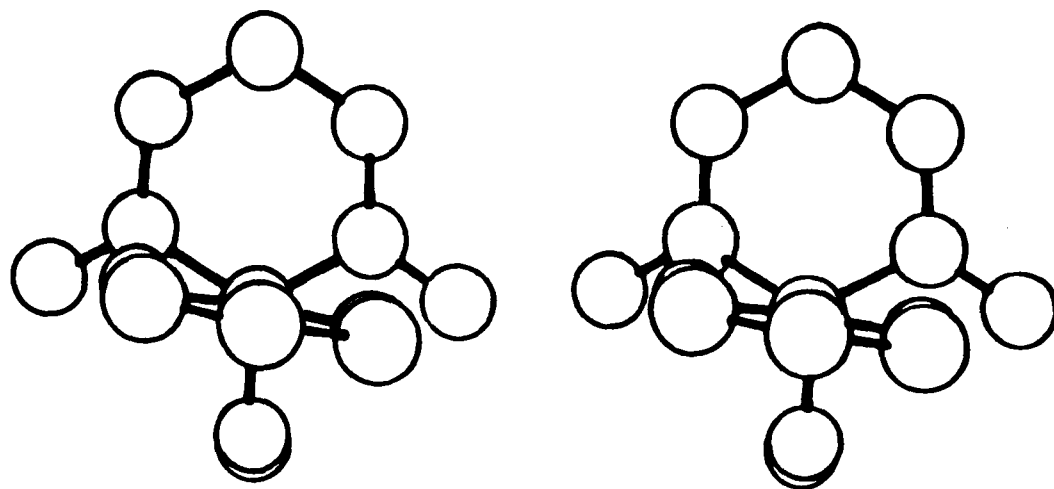


FIGURE 2: PSEUDOMIRROR SYMMETRY OF THE MOLECULE OF  
PRIMIDONE ABOUT THE MEDIAN LINE  $C(2)-C(5)$

conformation is exhibited by the pyrimidine ring (Table 6), with C(5) elevated towards the phenyl moiety. Corresponding bond angles and lengths on either side of the median line of the pyrimidine ring show no significant differences (Fig. 2) indicating high degree of ring symmetry. The phenyl ring is planar and the angle between this plane and the planar portion of the pyrimidine ring is  $87^\circ$  (Table 6). Two types of hydrogen bonds (Table 5) are involved to form infinite approximately parallel sheets of molecules (Fig. 3):

(i) N(1)-H(11) ... O(6) between molecules related by a centre of symmetry, N(1)-O(6) = 2.876 (5) Å

(ii) N(3)-H(31) ... O(4) between molecules related by two fold screw axis, N(3)-O(4) = 2.761 (5) Å.

Configurational similarities were observed in primidone, phenobarbital and phenytoin as compared by the Newman projections (Fig. 4) and intramolecular distances and dihedral angles of the common structural features (Table 7).

Table 1. Crystal data: primidone

Molecular formula	$C_{12}H_{14}N_2O_2$		
F.W.	218		
Crystal system	Monoclinic		
Habit form	Prismatic, elongated in b		
Space group	P2/c	$D_m$	$1.267 \text{ g cm}^{-3}$
a	12.245 (3) Å	$D_c$	$1.277 \text{ g cm}^{-3}$
b	7.088 (2)	F(000)	464
c	14.805 (4)	$\mu(\text{Cu K}\alpha)$	$10.47 \text{ cm}^{-1}$
$\beta$	$117.82 (1)^\circ$	$\lambda(\text{Cu K}\alpha_1)$	$1.54051 \text{ Å}$
$V_c$	$1136.45 \text{ Å}^3$	Crystal size 0.2 x 0.4 x 0.2 mm	
Z	4	$\omega$ axis	b

Table 2. Atomic parameters

(a) Refined positional and anisotropic thermal parameters for non-H atoms ( $\times 10^4$ ) with e.s.d.'s in parentheses.

Anisotropic temperature factor is of the form  $\exp \{-2\pi^2(h^2a^2U_{11} + k^2b^2U_{22} + l^2c^2U_{33} + 2klb^*c^*U_{23} + 2lhc^*a^*U_{31} + 2hka^*b^*U_{12})\}$ .

	x	y	z	$U_{11}$	$U_{22}$	$U_{33}$	$U_{12}$	$U_{13}$	$U_{23}$
N(1)	0.3603(2)	0.5216(3)	0.3801(2)	315(10)	310(10)	434(12)	79(9)	101(9)	6(9)
C(2)	0.2622(2)	0.4656(4)	0.2824(2)	359(13)	340(13)	448(14)	24(10)	152(11)	-55(12)
N(3)	0.1461(2)	0.5348(3)	0.2715(2)	296(11)	320(11)	706(15)	-47(9)	170(11)	-42(11)
C(4)	0.1351(2)	0.7042(3)	0.3048(2)	305(13)	313(12)	570(16)	46(10)	186(11)	98(11)
C(5)	0.2536(2)	0.8223(3)	0.3549(2)	311(12)	251(13)	403(13)	18(9)	143(10)	20(10)
C(6)	0.3631(2)	0.6914(3)	0.4194(2)	352(13)	299(12)	388(13)	7(10)	124(11)	28(10)
C(11)	0.2768(2)	0.9127(3)	0.2710(2)	392(13)	211(11)	432(14)	32(9)	198(11)	-9(10)
C(12)	0.1814(3)	0.9521(4)	0.1753(2)	566(18)	437(16)	460(15)	-28(13)	141(13)	69(13)
C(13)	0.2050(4)	1.0433(5)	0.1030(3)	1067(30)	526(19)	483(18)	75(20)	289(20)	103(16)
C(14)	0.3221(5)	1.0960(5)	0.1257(3)	1217(35)	478(18)	860(27)	118(20)	786(28)	140(18)
C(15)	0.4164(4)	1.0616(5)	0.2203(4)	766(25)	546(19)	1168(32)	106(18)	727(25)	187(20)
C(16)	0.3954(3)	0.9721(4)	0.2941(3)	437(16)	406(15)	723(20)	47(12)	323(15)	106(14)
C(21)	0.2415(3)	0.9822(4)	0.4200(2)	634(19)	298(13)	507(16)	78(13)	320(15)	23(12)
C(22)	0.2171(5)	0.9183(6)	0.5076(3)	1222(37)	516(20)	756(24)	118(23)	683(27)	14(19)
O(4)	0.0359(2)	0.7618(3)	0.2956(2)	358(11)	446(11)	1159(19)	98(9)	364(12)	75(12)
O(6)	0.4480(2)	0.7436(3)	0.5011(1)	526(11)	372(10)	475(11)	35(8)	-23(9)	-26(8)

Table 2. (Continued)

(b) Refined positional and isotropic thermal parameters for H atoms.  $\bar{U}_{iso}^2$  is the isotropic temperature factor in the expression  $B_{iso}^2 = 8\pi^2\bar{U}_{iso}$

	x	y	z	$\bar{U}_{iso}^2$ (x 10 <sup>4</sup> )
H(11)	0.4209(29)	0.4456(45)	0.4094(22)	162(74)
H(21)	0.2567(27)	0.3285(48)	0.2755(23)	213(75)
H(22)	0.2776(24)	0.5130(40)	0.2255(22)	110(66)
H(31)	0.0858(33)	0.4616(52)	0.2427(25)	295(90)
H(121)	0.0990(34)	0.9222(50)	0.1642(24)	303(87)
H(131)	0.1311(41)	1.0646(64)	0.0364(35)	589(121)
H(141)	0.3420(32)	1.1548(53)	0.0747(28)	393(96)
H(151)	0.4974(38)	1.0992(55)	0.2386(28)	384(98)
H(161)	0.4625(31)	0.9453(49)	0.3663(26)	304(86)
H(211)	0.1767(26)	1.0638(42)	0.3737(21)	115(65)
H(212)	0.3221(27)	1.0500(41)	0.4516(21)	126(67)
H(221)	0.1397(43)	0.8419(71)	0.4793(34)	616(139)
H(222)	0.2902(40)	0.8372(69)	0.5555(33)	588(127)
H(223)	0.2045(42)	1.0250(73)	0.5425(36)	688(136)

Table 3. Bond lengths and angles

Standard deviations are given in parentheses

(a) Bond lengths (Å) for the non-hydrogen atoms

N(1)-C(2)	1.441 (5)	C(6)-O(6)	1.230 (5)
N(1)-C(6)	1.329 (6)	C(11)-C(12)	1.387 (6)
C(2)-N(3)	1.441 (6)	C(11)-C(16)	1.390 (7)
N(3)-C(4)	1.329 (6)	C(12)-C(13)	1.393 (8)
C(4)-O(4)	1.223 (5)	C(13)-C(14)	1.359 (10)
C(4)-C(5)	1.540 (6)	C(14)-C(15)	1.361 (11)
C(5)-C(6)	1.543 (5)	C(15)-C(16)	1.392 (8)
C(5)-C(11)	1.533 (6)	C(21)-C(22)	1.532 (8)
C(5)-C(21)	1.540 (6)		

(b) Bond angles (°) for the non-hydrogen atoms

N(1)-C(2)-N(3)	110.0 (4)	C(6)-C(5)-C(11)	108.4 (3)
C(2)-N(3)-C(4)	122.1 (4)	C(11)-C(5)-C(21)	107.8 (3)
N(3)-C(4)-C(5)	115.8 (4)	C(11)-C(12)-C(13)	120.1 (5)
N(3)-C(4)-O(4)	122.2 (4)	C(12)-C(13)-C(14)	121.0 (6)
O(4)-C(4)-C(5)	122.0 (4)	C(13)-C(14)-C(15)	119.5 (5)
C(4)-C(5)-C(6)	108.8 (3)	C(14)-C(15)-C(16)	121.0 (6)
C(4)-C(5)-C(11)	109.0 (3)	C(15)-C(16)-C(11)	120.1 (5)
C(4)-C(5)-C(21)	111.0 (4)	C(16)-C(11)-C(12)	118.3 (4)
C(5)-C(6)-C(1)	116.0 (3)	C(16)-C(11)-C(5)	119.8 (4)

Table 3. (Continued)

C(5)-C(6)-O(6)	121.0 (4)	C(21)-C(5)-C(6)	111.7 (3)
C(6)-C(6)-N(1)	122.9 (4)	C(22)-C(21)-C(5)	114.8 (4)
C(6)-N(1)-C(2)	121.8 (3)		

(c) Bond lengths ( $\text{\AA}$ ) involving hydrogen atoms

H(11)-N(1)	0.85 (6)	H(151)-C(15)	0.94 (8)
H(21)-C(2)	0.98 (6)	H(161)-C(16)	1.02 (6)
H(22)-C(2)	1.00 (5)	H(211)-C(21)	0.96 (5)
H(31)-N(3)	0.84 (7)	H(212)-C(21)	1.00 (6)
H(121)-C(12)	0.97 (7)	H(221)-C(22)	1.00 (10)
H(131)-C(13)	0.99 (9)	H(222)-C(22)	1.02 (9)
H(141)-C(14)	0.99 (7)	H(223)-C(22)	0.97 (10)

(d) Bond angles involving hydrogen atoms ( $^{\circ}$ )

H(11)-N(1)-C(2)	117 (4)	H(151)-C(15)-C(14)	122 (4)
H(11)-N(1)-C(6)	121 (4)	H(151)-C(15)-C(16)	188 (4)
H(21)-C(2)-N(1)	112 (3)	H(161)-C(16)-C(15)	124 (4)
H(21)-C(2)-N(3)	107 (3)	H(161)-C(16)-C(11)	116 (4)
H(21)-C(2)-N(22)	107 (5)	H(211)-C(21)-C(5)	107 (3)
H(22)-C(2)-N(1)	110 (3)	H(211)-C(21)-C(22)	111 (3)
H(22)-C(2)-N(3)	112 (3)	H(211)-C(21)-H(212)	111 (4)
H(22)-N(2)-C(2)	116 (4)	H(212)-C(21)-C(5)	107 (3)
H(31)-N(3)-C(4)	122 (4)	H(212)-C(21)-C(22)	107 (3)
H(121)-C(12)-C(11)	117 (4)	H(221)-C(22)-C(21)	109 (5)
H(121)-C(12)-C(13)	123 (4)	H(221)-C(22)-H(222)	110 (8)
H(131)-C(13)-C(12)	115 (5)	H(221)-C(22)-H(223)	107 (7)
H(131)-C(13)-C(14)	125 (5)	H(222)-C(22)-C(21)	107 (4)
H(141)-C(14)-C(13)	122 (4)	H(222)-C(22)-H(223)	112 (7)
H(141)-C(14)-C(15)	118 (4)	H(223)-C(22)-C(21)	111 (5)

Table 4. Torsion angles ( $^{\circ}$ ) determined for primidone

The sign of the angle A-B-C-D is positive when a clockwise rotation about B-C is required to bring A-B-C into coincidence with B-C-D, viewed along B-C.

A	B	C	D	
C(2)	-N(1)	-C(6)	-C(5)	-1
C(2)	-N(3)	-C(4)	-C(5)	+1
C(2)	-N(1)	-C(6)	-O(6)	-2
C(2)	-N(3)	-C(4)	-O(4)	0
N(1)	-C(2)	-N(3)	-C(4)	+141
N(3)	-C(2)	-N(1)	-C(6)	-40
N(1)	-C(6)	-C(5)	-C(4)	+38
N(3)	-C(4)	-C(5)	-C(6)	+141
N(1)	-C(6)	-C(5)	-C(11)	+81
N(3)	-C(4)	-C(5)	-C(11)	-79
N(1)	-C(6)	-C(5)	-C(21)	+19
N(3)	-C(4)	-C(5)	-C(21)	-18
C(4)	-C(5)	-C(6)	-O(6)	+37
C(6)	-C(5)	-C(4)	-O(4)	+142
C(11)	-C(5)	-C(4)	-O(4)	-80
C(11)	-C(5)	-C(6)	-O(6)	+82
C(11)	-C(5)	-C(21)	-C(22)	0
C(12)	-C(11)	-C(5)	-C(4)	+27
C(12)	-C(11)	-C(5)	-C(6)	+146
C(12)	-C(11)	-C(5)	-C(21)	-86
C(16)	-C(11)	-C(5)	-C(4)	+21
C(16)	-C(11)	-C(5)	-C(6)	+140
C(16)	-C(11)	-C(5)	-C(21)	-81
C(21)	-C(5)	-C(4)	-O(4)	+19
C(21)	-C(5)	-C(6)	-O(6)	-20
C(22)	-C(21)	-C(5)	-C(4)	+60
C(22)	-C(21)	-C(5)	-C(6)	-62

Table 5. Hydrogen-bond distances and angles

X	H	Y	X ... Y ( $\text{\AA}$ )	$\angle$ X-H ... Y ( $^{\circ}$ )
N(1)	H(11)	O(6')	2.876 (5)	169.6 (3)
N(3)	H(31)	O(4'')	2.761 (5)	165.4 (3)

Symmetry code: (i)  $1-x, 1-y, 1-z$ . (ii)  $-x, -\frac{1}{2} + y, \frac{1}{2} - z$ .



Table 6. Least-squares planes

Plane	Atom	R.m.s. displacement or deviation (Å)	†Equation of plane
Pyrimidine	A N(1)		$0.161X^1 + 0.422Y^1 + 0.892Z^1 + 0.990 = 0$
	N(3)	0.004	
	C(4)		
	C(6)		
	C(2)*	0.447	
	C(5)*	0.498	
	C(4)*	-0.338	
	O(6)*	-0.387	
B	N(3)		$0.210X^1 - 0.393Y^1 + 0.895Z^1 - 1.695 = 0$
	C(4)	0.001	
	O(4)		
	C(5)		
C	N(1)		$0.492X^1 + 0.355Y^1 - 0.795Z^1 - 0.394 = 0$
	C(6)	0.003	
	O(6)		
	C(5)		
D	C(11)		$0.154X^1 - 0.883Y^1 - 0.442Z^1 + 6.34 = 0$
	C(12)		
	C(13)	0.009	
	C(14)		
	C(15)		
	C(16)		

\*Not included in the calculation of the equation of the plane.

†The equations are defined with respect to orthogonal axes  $X^1(a^*)$ ,  $Z^1(c)$ ,  $Y^1$  expressed in Å.

Table 7. Intramolecular distances and dihedral angles

(a) Intramolecular oxygen-ring centroid/oxygen distances ( $\text{\AA}$ )<sup>O</sup>  
in primidone, phenobarbital and phenytoin

Oxygen	Ring centroid or oxygen	Distance ( $\text{\AA}$ ) <sup>O</sup>
Primidone		
O(4)	C(11) to C(16)	4.46
O(6)	C(11) to C(16)	4.39
O(4)	O(6)	4.51
Phenobarbital		
O(4)	C(11) to C(16)	4.34
O(6)	C(11) to C(16)	4.27
O(2)	C(11) to C(16)	5.42
O(4)	O(6)	4.79
Phenytoin		
O(6)	C(8) to C(13)	5.68
O(7)	C(8) to C(13)	3.97
O(6)	C(14) to C(19)	5.51
O(7)	C(14) to C(19)	4.23
O(6)	O(7)	4.56

(b) Dihedral angles ( $^{\circ}$ ) in primidone, phenobarbital and phenytoin

Primidone		
Phenyl-pyrimidine	N(1), N(3), C(4), C(6)	87 <sup>O</sup>
Phenobarbital		
Phenyl-pyrimidine	N(1), C(2), N(3), C(4), C(6)	86
Phenytoin		
Hydantoin-phenyl	C(8) to C(13)	114
Hydantoin-phenyl	C(14) to C(19)	113
Phenyl	C(8) to C(13)-phenyl C(14) to C(19)	90

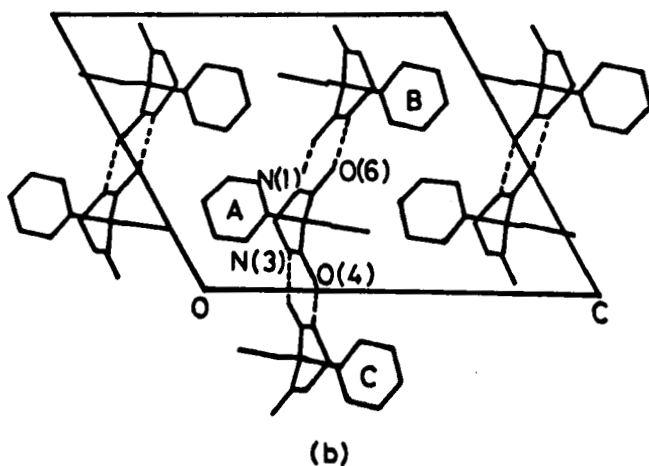
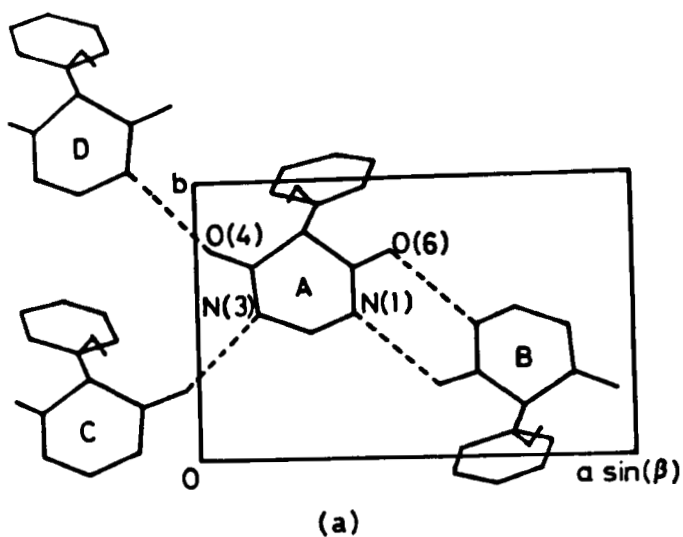


FIGURE 3: HYDROGEN -BOND SCHEME VIEWED (a) ALONG  $c$  AXIS, AND (b) ALONG  $b$  AXIS (5)

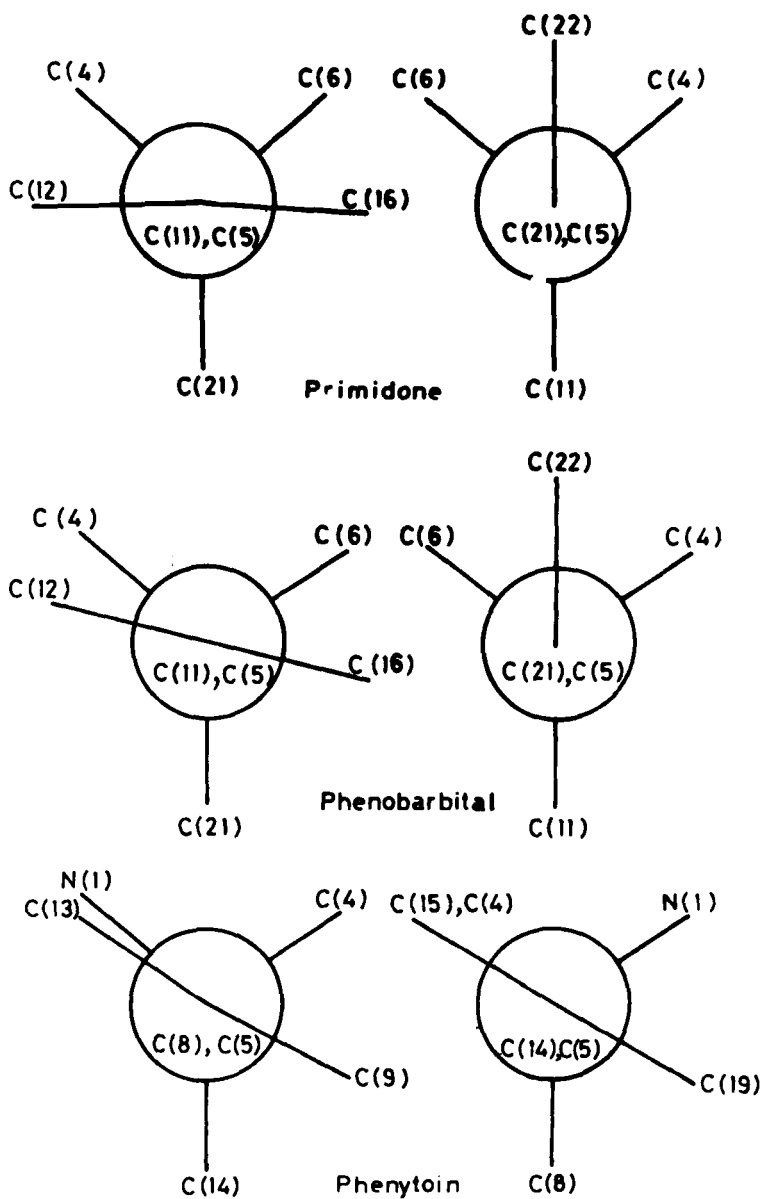


FIGURE 4. NEWMAN PROJECTIONS SHOWING THE CONFIGURATION ABOUT C<sub>(5)</sub> IN PRIMIDONE, PHENOBARBITAL & PHENYTOIN(5)

## 2.5 Spectral Properties

### 2.5.1 Ultraviolet Spectrum

The ultraviolet absorption spectrum of primidone in methanol was obtained on a Cary 219 spectrophotometer and is shown in Figure 5. The spectrum is characterized by maxima at 264, 258, 252 and 227 nm.

### 2.5.2 Infrared Spectrum

The infrared absorption spectrum of primidone dispersed in potassium bromide was recorded on a Pye Unicam SP 1025 spectrometer and is presented in Figure 6. The characteristic bands and their assignment are listed below.

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignment</u>
3300-3200	N-H stretch (H-bonded).
2990	Aromatic C-H stretch
1720	Amide C = O
1620, 1600, 1500	Aromatic C $\cdots$ C stretch
780, 765	Aromatic C-H out-of-plane bending.

### 2.5.3 <sup>1</sup>H Nuclear Magnetic Resonance (<sup>1</sup>H NMR) Spectrum

Primidone solution in DMSO-D<sub>6</sub> was used to obtain the <sup>1</sup>H NMR spectrum on Varian-T60A NMR spectrometer with TMS as the internal standard. The spectrum is shown in Figure 7, and assignment of the chemical shifts to the different protons is summarized below.

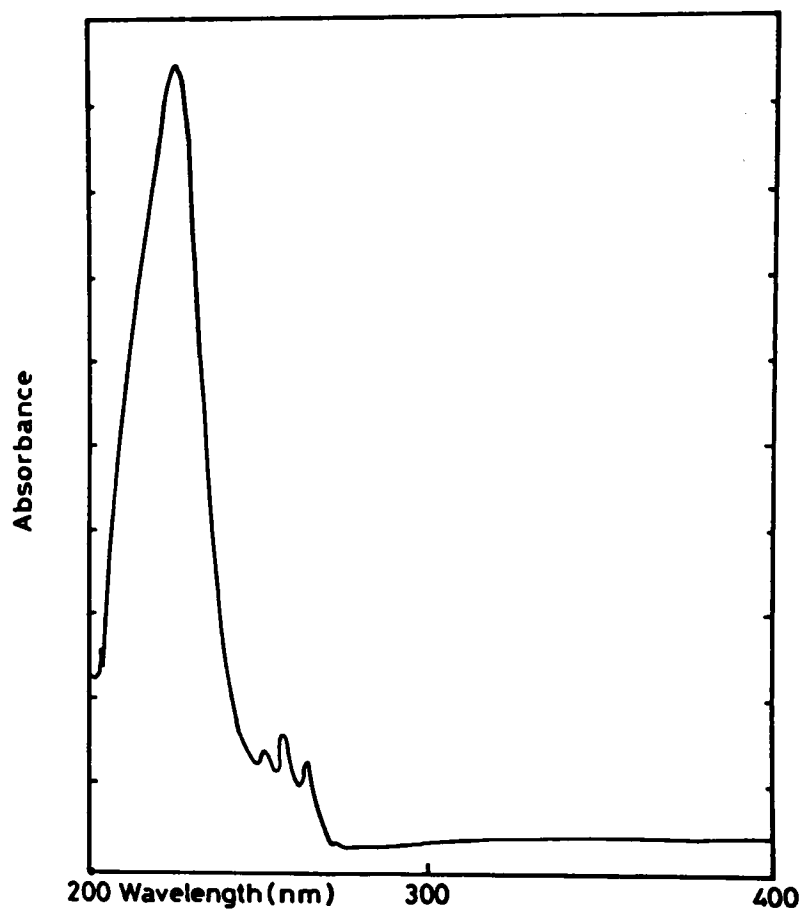


FIGURE 5: ULTRAVIOLET SPECTRUM OF PRIMIDONE IN METHANOL.

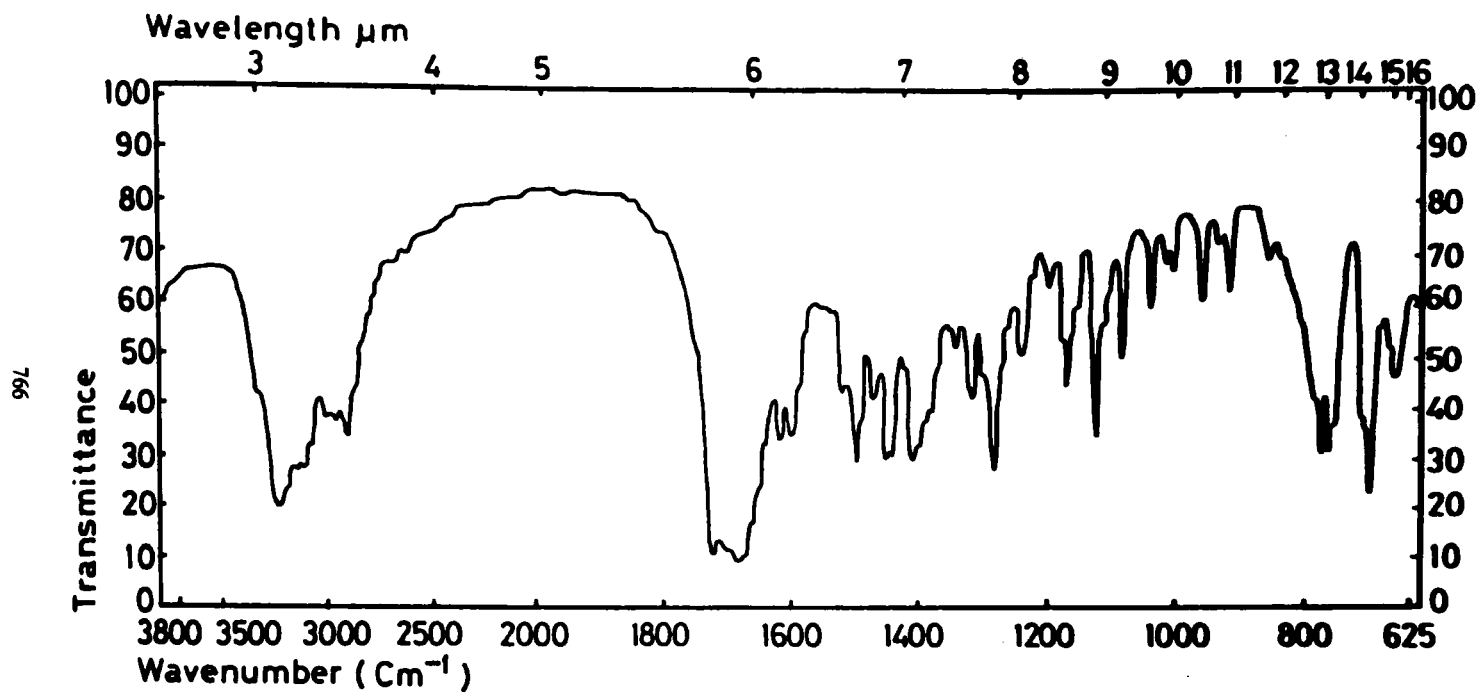


FIGURE 6: INFRARED SPECTRUM OF PRIMIDONE, KBr DISC.

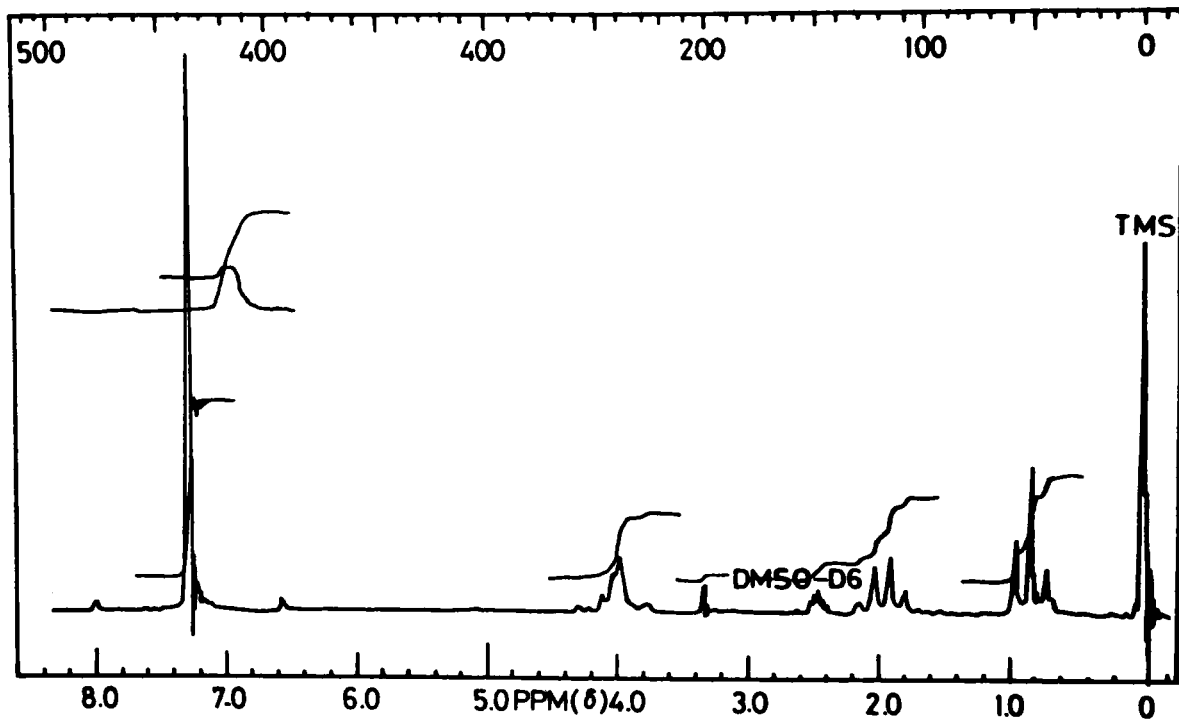


FIGURE 7:  $^1\text{H}$  NMR SPECTRUM OF PRIMIDONE IN  $\text{DMSO}-d_6$  USING TMS AS INTERNAL STANDARD.

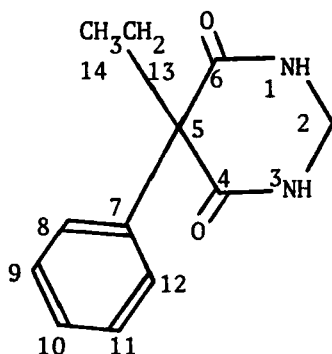


<u>Chemical shift (<math>\delta</math>)</u>	<u>Multiplicity</u>	<u>Proton Assignment</u>
0.83	triplet	$\text{CH}_3$ protons
1.99	quartet	$\text{CH}_3\text{CH}_2$ protons
4.04	multiplet	$  \begin{array}{c}  \text{H} \\    \\  \text{-N} \quad \diagdown \\  \quad \quad \text{CH}_2 \\  \quad \quad / \\  \text{-N} \quad \diagup \\    \\  \text{H}  \end{array}  $ protons
7.30	multiplet	Aromatic protons
8.64	doublet	$\text{NH}$ protons

#### 2.5.4 $^{13}\text{C}$ Nuclear Magnetic Resonance ( $^{13}\text{C}$ NMR) Spectrum

The  $^{13}\text{C}$  NMR spectra of primidone in DMSO- $\text{D}_6$  using TMS as internal standard are obtained using a Jeol FX 100 90 MHz spectrometer at ambient temperature. Figure 8 and Figure 9 show the  $^1\text{H}$ -decoupled and off-resonance spectra, respectively. The carbon chemical shifts, assigned on the basis of the off-resonance splitting pattern and the theories of chemical shifts, are presented in Table 8.

Table 8. Carbon chemical shifts and assignments



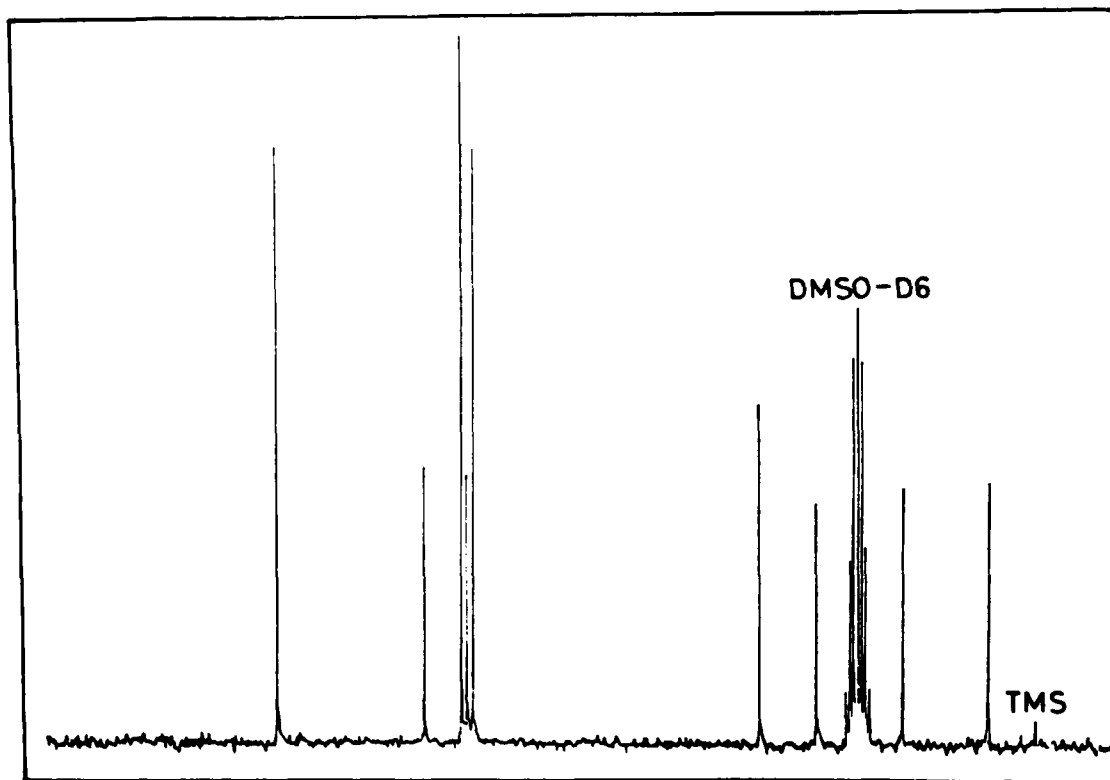


FIGURE 8: NOISE-DECOUPLED  $^{13}\text{C}$  NMR SPECTRUM OF PRIMIDONE IN DMSO-D6 USING TMS AS INTERNAL STANDARD.

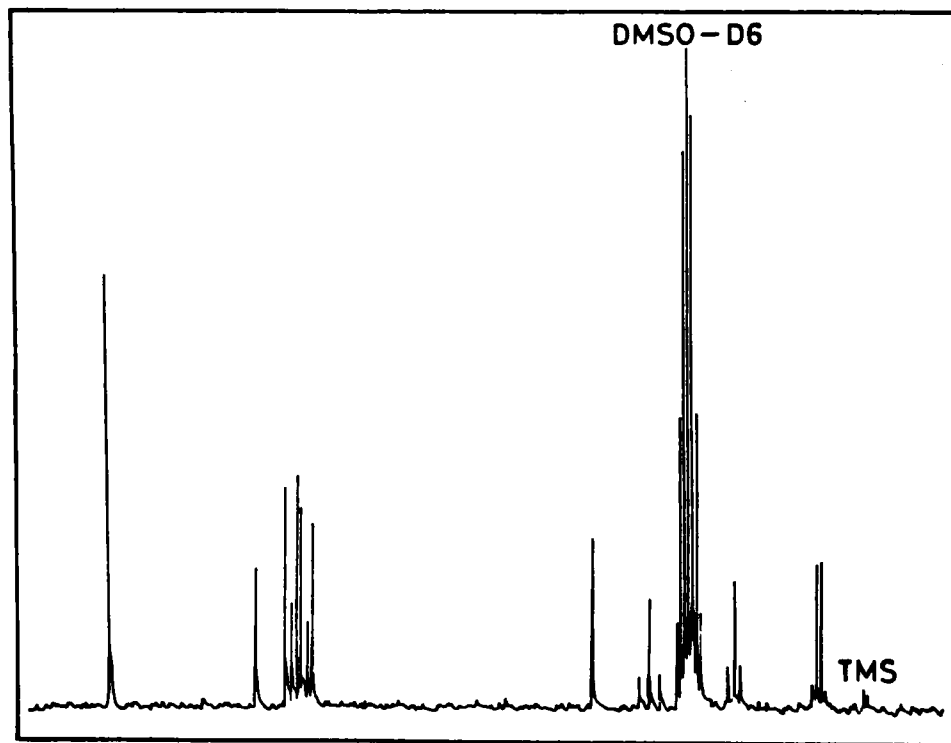


FIGURE 9: OFF-RESONANCE  $^{13}\text{C}$  NMR SPECTRUM OF PRIMIDONE IN DMSO-D6 USING TMS AS INTERNAL STANDARD.

<u>Chemical shifts (<math>\delta</math>)</u>	<u>Multiplicity</u>	<u>Carbon Assignment</u>
10.33	Quartet	C <sub>14</sub>
29.36	Triplet	C <sub>13</sub>
48.26	Triplet	C <sub>2</sub>
61.00	Singlet	C <sub>5</sub>
126.05	Doublet	C <sub>8</sub> , C <sub>12</sub>
127.34	Doublet	C <sub>10</sub>
128.63	Doublet	C <sub>9</sub> , C <sub>11</sub>
137.08	Singlet	C <sub>7</sub>
170.14	Singlet	C <sub>4</sub> , C <sub>6</sub>

#### 2.5.5 Mass Spectrum

The 70 eV electron impact (EI) mass spectrum of primidone, shown in Figure 10, was obtained on a Varian MAT 311 mass spectrometer using ion source of  $10^{-6}$  Torr, ion source temperature of 180°C and an emission current of 300 A. The molecular ion peak is detectable at m/e 218 and the base peak at m/e 146. A proposed mechanism of the fragmentation pattern and the mass/charge ratios of the major fragments are shown in Scheme 1. The chemical ionization (CI) spectrum was obtained on a Finnigan 4000 mass spectrometer using methane as a reagent with ion electron energy of 100 eV, ion source pressure of 0.3 Torr, ion source temperature of 150°C and emission current of 300 A. The spectrum presented in Figure 11, is dominated by a quasi-molecular ion ( $M + 1$ ). Peaks appearing at m/e 247 and m/e 259 are attributable to the transfer of carbocations from the carrier gas. The mass spectral assignments of the prominent ions under CI conditions are shown below.

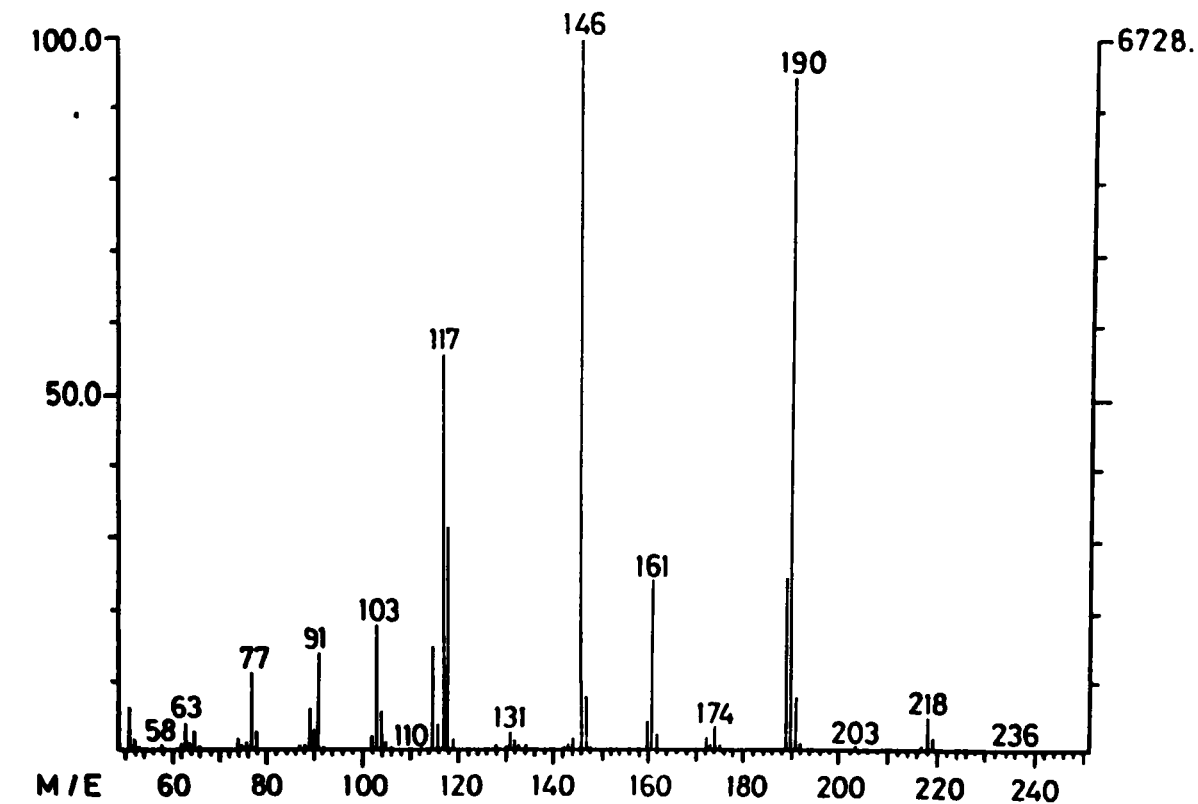


FIGURE 10: ELECTRON IMPACT MASS SPECTRUM OF PRIMIDONE.

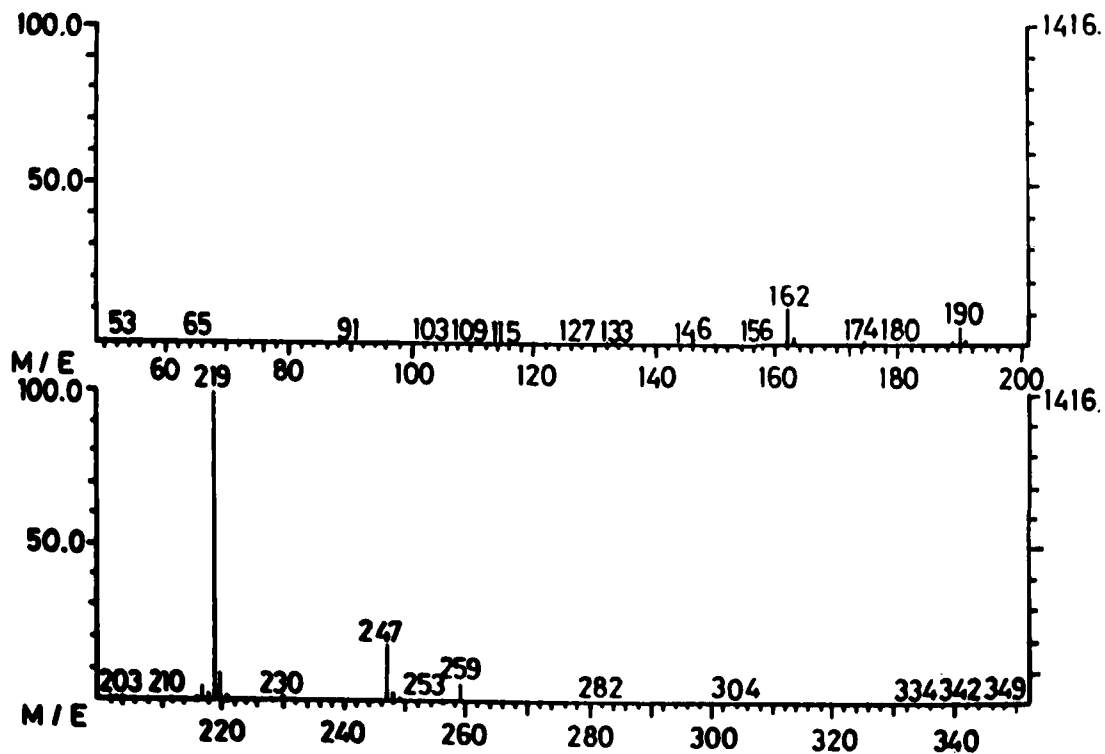
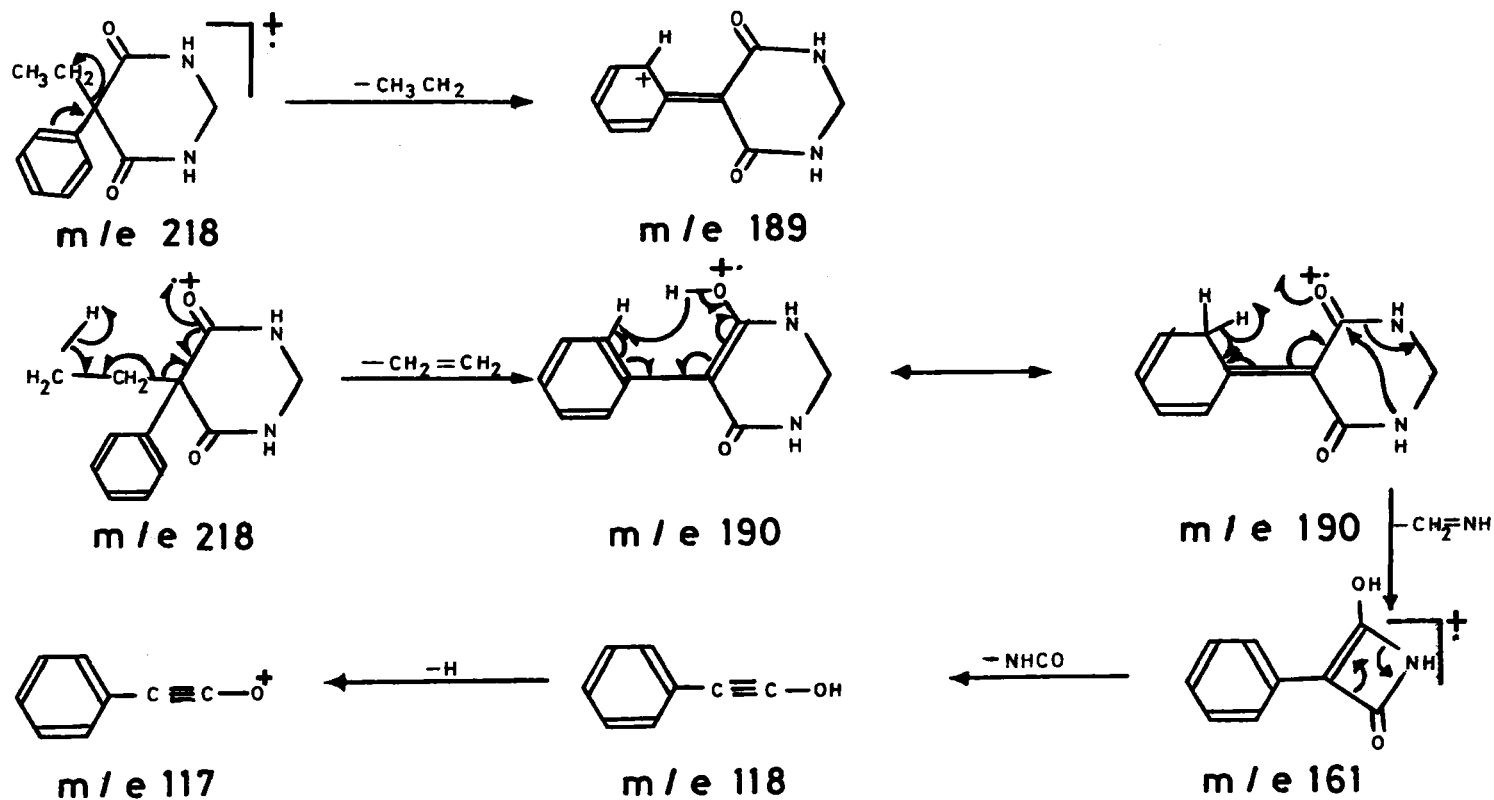
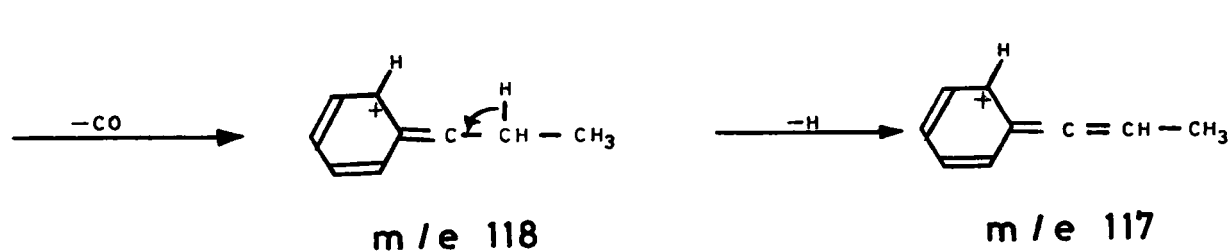
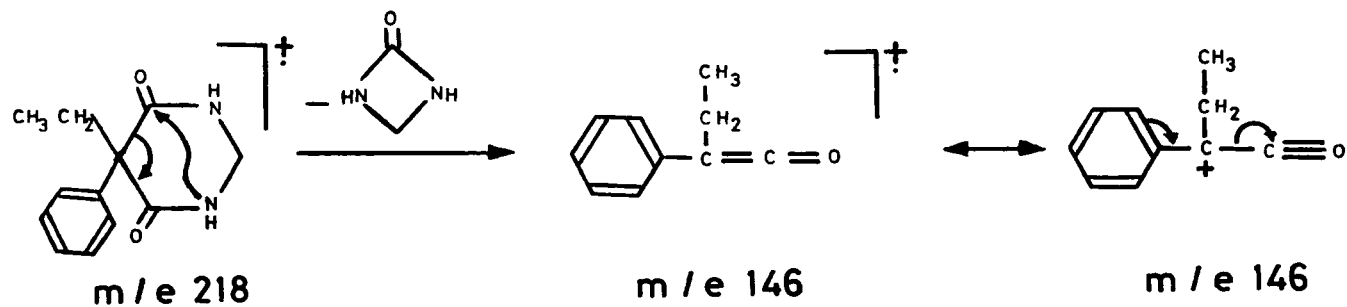


FIGURE 11: CHEMICAL IONISATION MASS SPECTRUM OF PRIMIDONE.



Scheme 1: Proposed mechanism of fragmentation of primidone.



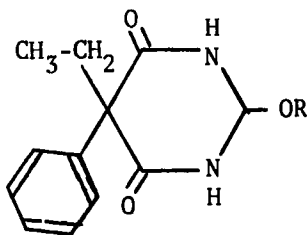
Scheme 1: continued.



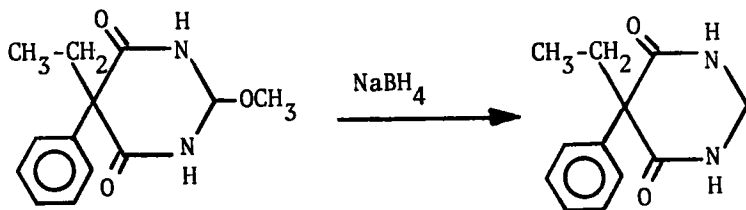
<u>m/e</u>	<u>Species</u>
162	$[\text{C}_9\text{H}_7\text{NO}_2 + \text{H}]^+$
219	$\text{MH}^+$
220	$\text{MH}_2^+$
247	$[\text{M} + \text{C}_2\text{H}_5]^+$
259	$[\text{M} + \text{C}_3\text{H}_5]^+$

### 3 Synthesis

A recent method for the manufacture of primidone has been described by Squine and Warren (6). Hexahydropyrimidinediones (Structure 1) are reduced with metal hydrides. Thus, when  $\text{NaBH}_4$  was added to a suspension of the methoxy derivative of 1 in THF and the mixture refluxed for 3 hr gave primidone.



I



## 4 Methods of Analysis

### 4.1 Nitrogen Determination

Helboe and Holch (7) have determined primidone by the use of Kjeldahl method. The digestion profiles (% Nitrogen found as a function of time) were determined on some common drug including primidone; digestion was in most cases completed in 5 minutes, and no loss occurred when the digestion was continued for two hours. A ruggedness test was made on the proposed method. Its unsatisfactory results are discussed. The size of the hole in the absbestos plate of the burner was identified as a source of possible error. After correction, a repeated ruggedness test was successful.

### 4.2 Polarographic Methods

The polarographic determination of primidone and some other drugs have been reported (8). The drug was determined in human plasma by extraction into chloroform-methyl acetate (60:40), nitration, thin-layer chromatography on silica gel with chloroform-isopropanol - 25%  $\text{NH}_3$  (45:45:10) and polarographic determination of the eluted derivatives. The limit of sensitivity was 3.5 - 5.5  $\mu\text{g/ml}$ .

### 4.3 Spectrophotometric Methods

Primidone in powder and tablet forms was quantitatively determined by ultraviolet spectrometry (9). The drug was subjected to optical density measurement at 258 nm and quantitatively determined by comparison of the experimental results with a calibration curve. An alcohol-water mixture as solvent gave more accurate results with primidone powder and ethanol alone gave more accurate results with the drug tablets than did a 1% sodium hydroxide solution.

Jeremic and Nikolic (10) published an improved ultraviolet spectrometric method for routine barbiturate monitoring of the drugs in serum and cerebrospinal fluids. The method can be used for monitoring primidone. The basis for the procedure is the Goldbaum's method (Anal. Chem. 24 1604, 1952). This method is improved with several modifications including a single extraction technique with dichloro-ethane, which has produced a very simple but still accurate method.

Proton magnetic resonance spectra of primidone and other drugs were studied (11) and the chemical displacement  $\delta$  (in ppm) and coupling constants  $J$  (in Hz) recorded.

#### 4.4 Immunoassays

Pippenger (12) has presented an overview on the homogenous immunoassays for the quantitative determination of some anticonvulsant agents including primidone in serum or plasma. He described a procedure which has the advantages of radioimmunoassay but does not require utilization of radioactive compounds. The accuracy was not affected by slight hemolysis and none of the compounds had cross-reactivity with their respective metabolites.

McClellan et al (13) adapted the enzyme-multiplied immuno-technique (EMIT), that involves use of glucose 6-phosphate dehydrogenase as the enzyme label, to a fully mechanized kinetic enzyme analyser for analysis of primidone and other anticonvulsant agents. This procedure, compared with a previously reported gas-chromatographic procedure, gave a weighted regression line forced through the origin of  $Y = (1.04 \pm 0.06)X$  for primidone. Run-to-run assay coefficient of variation was 6% for primidone based on the mean of triplicate determinations of a sample with a mid-range concentration.

#### 4.5 Chromatographic Methods

##### 4.5.1 Thin-Layer Chromatography

Quantitative thin-layer chromatography determination of primidone in biological fluid has been reported (14). A rapid and quantitative determination of the drug in biological fluids was developed consisting of extracting the drug with chloroform, evaporating to dryness and spilling a chloroform-acetic acid solution of the residue on a thin-layer of silica gel. Quantitation was achieved by comparing the areas under the peaks obtained from scanning the chromatographic plates in a spectrophotometer. The limit of detection was 1  $\mu\text{g/ml}$  plasma for primidone.

##### 4.5.2 Gas Chromatography

Cooper et al (15) described a gas-liquid chromatography method for the isolation, identification and

quantitation of primidone in whole blood. The blood sample is extracted with ether at pH 2 and then separately but simultaneously gas-liquid chromatographed on a selective and nonselective column. Retention data and clinical results from patients are given.

Fricke (16) presented a method for the analysis of primidone using simple extractions and semiautomated gas-liquid chromatography, using Dexsil 300 as the liquid phase and an automatic sample injector. The author has made a comparison between the results obtained by this method and the official or other applicable methods. By this method, content uniformity analyses can be made. The extraction and chromatographic conditions were standardized to make possible a successful interlaboratory study.

Simultaneous determination of primidone and other drugs in small samples of blood by gas-liquid chromatography have been reported (17). Acidified blood was extracted with ether, the extract was evaporated to dryness, redissolved in ether containing methyltestosterone (internal standard) was chromatography determined on an SE 3 column using a flame ionization detector. Recovery was 70.7% at a 2 mg/100 ml concentration.

Goudie and Burnett (18) have used a heated nitrogen detector in a gas chromatography method for the simultaneous determination of primidone and other drugs in serum following therapeutic dosages. The internal standard used was an analog of phenytoin. The drug was methylated in the injection point heater of the chromatograph. The N detector was highly selective and allowed the use of a direct extraction procedure.

Primidone and other drugs have been simultaneously determined in serum, by use of gas-liquid chromatography (19). The method is simple and sensitive. The methylated derivative of the drug was well resolved as was 5-(p-methylphenyl)-5-phenyl hydantoin, (the internal standard). In this procedure, an ion exchange resin for separation of the drug from the serum is used. The proposed procedure requires only 1 ml of serum and can be done in less than one hour.

St. Onge et al. (20) reported the use of a preparative instrument for excretion and gas chromatography determination of primidone. A microprocessor-controlled automatic extractor with which lipophilic components may be extracted from physiological fluids by means of a selective, solid phase extraction cartridge, and the extract is presented in dry form for subsequent analysis. The method for the determination of primidone by the above instrument and analysis after flash-heater methylation, in a gas-chromatograph equipped with N-sensitive detector was used. The glass column was packed with 3% OV-17 on Chromosorb W-HP 100/120 and He was used as the carrier gas. Recovery was 90-102%.

A gas chromatographic method (21) for the determination of primidone and other drugs in the same extract of serum was reported. Saturated solution of ammonium sulphate is added to 1 ml serum followed by extraction with chloroform. The organic base was separated and evaporated. The residue is dissolved in 100  $\mu$ l ethyl acetate: acetic acid mixture (100:1) for gas chromatography. The gas chromatography was performed under isothermal conditions without derivatization using Doxsil 300 as the stationary phase. The recovery was from 93.5 to 111%.

Plotczyk (22) have examined the feasibility of using fused-silica capillary chromatography for the routine analysis of several common drugs including primidone. Cold on-column injection with cross-linked polysiloxane deactivated columns produced linear quantitation from 1 to 100 ng with precisions of 0.1 to 2% for the drugs.

Hulshoff et al. (23) have reported gas-chromatographic alkylation studies on primidone and other anticonvulsants. The alkylation of the drug with n-alkyl iodide in N,N-dimethylacetamide with tetramethylammonium hydroxide was investigated by gas chromatography. Primidone was converted with  $\text{CH}_3\text{I}$  and butyl iodide into a major derivative and a minor one. Derivatives were identified by mass spectrometry and by  $^1\text{H}$  NMR and C-13 NMR.

Latham and Varlow (24) described a simultaneous quantitative gas-chromatographic analysis of primidone, ethosuximide, phenobarbital and diphenylhydantoin

from 1 ml of serum. The extraction procedure was common to all four drugs and the internal standards. Isothermal gas chromatographic analysis of the extracts resulted in well resolved peaks for the underivatized quantitation of ethosuximide and phenobarbital. Primidone and diphenylhydantoin, however, were determined as methylated derivatives. Newly packed and conditioned column produced mean coefficients of variation for the assay of each drug of less than 7%. The value rises to less than 10% if the technique had been in continuous use for 3 months. The advantage of quantitation relative to peak area ratios rather than peak height ratios was significant only for the assay of phenobarbital.

Bannon and Vinet (25) reported a modified EMIT procedure for the analyses, in serum, of primidone and three other antiepileptic drugs. The EMIT drugs assays adapted to a Bichromatic Analyser and compared with a gas chromatographic procedure. Day-to-day precision for all the drugs tested is reported to be excellent with coefficients of variation averaging 5% in the therapeutic range of concentrations. Results for sera analysed by this procedure and by a gas chromatographic method, showed slight differences between the 2 methods for primidone and carbamazepine, which do not significantly modify the clinical interpretation of the results. The procedure is claimed to greatly reduce the operation costs.

A gas chromatographic method for the determination of anticonvulsant drugs in serum was described (26). The drugs were extracted with ether : toluene (3 : 7 v/v) mixture. The procedure did not require solvent evaporation. The coefficient of variance 1.2% for 9.6 µg/ml primidone.

Sun and Walwick (27) correlated a gas-chromatographic assay with an enzyme immunoassay. Serum specimens from primidone-treated patients were assayed by EMIT and a gas-chromatographic procedures. Results by the two methods agreed well, suggesting that the two procedures could be used interchangeably. The correlation coefficient was 0.98 for the 94 specimens studied and the least squares values of the slope and intercept were 0.97 and 0.51 mg/L respectively.

Kapetanovic and Kupferberg (28) reported GC and GC-mass spectrometric determination of primidone in urine and its metabolites, phenobarbital and p-hydroxyphenobarbital in plasma, urine and hepatic microsomes. The compounds were determined in biological fluids following derivatization by a modification of a previously reported method. The major modification involves the use of MeCN instead of N,N-dimethylacetamide as the aprotic solvent. EtI was used for derivatization with plasma and hepatic microsomal extracts and PrI with urinary extracts. GC N-selective detection and/or selected ion monitoring were used for quantitation of the derivatives. The technique was applied using rats and human subjects.

Strauss and Hertel (29) applied a microcomputer-directed-mass spectrometer as a compound-selective detector for GC to the determination of primidone and other anticonvulsants in blood serum and urine. The GC-mass spectrometric system utilizes reverse spectral search and retention time screening to provide a high degree of compound specificity. Computer control of instrument operation, and of data acquisition, analysis and printout allows technologists to obtain highly reliable, precise quantitation using relatively crude sample preparations in short chromatography times.

A microassay for primidone and its metabolites phenobarbital, phenylethylmalondiamide and p-hydroxyphenobarbital, in human serum saliva, breast milk and tissues by GC-mass spectrometry using selective ion monitoring has been developed by Nau *et al* (30). The determinations include both free and conjugated drugs. Methyl analogs of primidone, phenobarbital and phenylethylmalondiamide were used as internal standards. Following the addition of the internal standards and of saturated  $(\text{NH}_4)_2\text{SO}_4$ , the samples were extracted twice with  $\text{EtOAc}/\text{C}_6\text{H}_6$  (20:80). Half the extracts was ethylated by Greeley's method for the analysis of primidone, phenobarbital and hydroxyphenobarbital while the other half was trimethylsilylated for the determination of primidone and phenylethylmalondiamide. The derivatized extracts were analysed by GC-mass spectrometry. The recoveries of the drugs were between 80 and 93%. The relative standard deviations were between 3.2 and 5.9%. These methods have been used to study the placental transfer and neonatal disposition

of primidone and its metabolites in the human.

#### 4.5.3 Liquid Chromatography

Mulard (31) has reported the biological application of automatic liquid-liquid extraction and liquid phase chromatography. Liquid-liquid extraction and liquid chromatography were improved by using third generation Technicon modules and a module for evaporation to dryness. The apparatus was used for the determination of primidone and other drugs in blood with good recovery and reproducibility.

#### 4.5.4 High-Pressure Liquid Chromatography

Van der Wal et al (32) have described two procedures for fully automated analysis of several drugs, including primidone in serum by using high-performance liquid chromatography, with on-line pretreatment (solvent extraction) of the sample. The FAST-LC system (Technicon instruments) was used for the assay of primidone in a mixture of other drugs. The rate of sample analysis was 12/hour for primidone. The precision of the assay was 3% (relative standard deviation) and only 75  $\mu$ l sample was required.

Dong and Dicesave (33) have reported a rapid analysis for primidone among some other abused drugs by liquid chromatography. A 2-4 fold in chromatography time was achieved for primidone and other drugs by using short columns packed with 305  $\mu$ m particles of silica, C<sub>8</sub> or C<sub>18</sub> bonded phases. The drug was separated in 2 minutes on a 5- $\mu$ m C<sub>18</sub> bonded-phase column. The flow rate was 4 ml/minutes and detection was at 195 nm.

Simultaneous determination of several compounds including primidone in serum by HPLC has been described (34). The serum is extracted with ethyl acetate at pH 3.9 and the dried extract is dissolved in 70% ethanol in water and an aliquot is injected into a Hewlett-Packard 1084 B liquid chromatograph. A reversed-phase (RP-8) column is used with methyl cyanide and water as the mobile phase. The eluted drugs are detected at 207 nm.

A reversed-phase high-pressure liquid chromatography method for the determination of primidone and other drugs in blood has been described (35). The drug was



extracted into methanol/chloroform solvent mixture. The extract was evaporated, reconstituted with mobile phase (chloroform and methanol in phosphate buffer), and separated by the reversed-phase HPLC; the effluent was monitored at 195 and 254 nm.

Soldin and Hill (36) described a micro HPLC method for the determination of primidone, phenobarbital and three other anticonvulsant agents in serum. The method involves precipitation of proteins with an acetonitrile solution containing cyheptamide as an internal standard, and reverse-phase chromatography on a  $\mu$ Bondapak C18 - containing column. The drugs are eluted with an equivolume mixture of potassium phosphate buffer and acetonitrile, detected by their absorbance at 200 nm, and quantitated by measuring peak areas. Analysis recoveries for the five drugs varied from 92% to 102% with coefficients of variation between 2.8% and 8.2% for therapeutic and toxic concentrations. The results of this method compare favorably with results obtained by GLC.

Blood serum levels of anticonvulsants were determined using HPLC, by Soldin and Hill (37), in children undergoing anticonvulsant therapy. Waters  $\mu$ Bondapak C-18 column was used. Elution carried out using a mixture of  $K_3PO_4$  buffer and acetonitrile. Drugs were detected bichromatically by measuring absorbance at both 200 nm and 254 nm. Recovery of primidone was 80%.

A HPLC method has been described by Haraguchi and Hata (38) for the simultaneous quantitation of primidone, phenobarbital, phenytoin and carbamazepine in serum, saliva, cerebrospinal fluid and urine.

Kraak *et al* (39) applied on-column concentration of deproteinized serum to HPLC determination of anticonvulsants. They investigated reverse-phase HPLC for the determination of anticonvulsants by direct injection of deproteinized serum. For simultaneous determination of primidone, phenobarbital, carbamazepine and diphenylhydantoin directly in deproteinized serum, octyl-modified silica as stationary phase was found suitable when 0.1 M phosphate buffer (pH 3.0) was used as the mobile phase.

Van Veldhuizen and Hartmann (40) identified and quantitated some of the most commonly abused sedatives and hypnotics using 100  $\mu$ l serum. Primidone and 14 other drugs are extracted in  $\text{CHCl}_3$ -EtOH solvent containing either 5-(p-methylphenyl)-5-phenylhydantoin or hexobarbital as an internal standard. The extract evaporated, reconstituted with mobile phase and injected into a high-performance liquid chromatograph in conjunction with a radial compression separation system. Peak heights are measured at 195 nm and 254 nm and sensitivity for all drugs is 1  $\mu$ g/ml. Day to day precision obtained coefficients of variation ranged from 4.5 to 10.4%

A HPLC procedure for the determination of primidone in tablets was reported by Roberts (41). Aqueous methanol is used as mobile phase and a Nucleosil C8 column is used for separation with measurement at 254 nm.

## 5 Pharmacokinetics

### 5.1 Absorption

Precise plasma concentrations required for therapeutic effects are unknown (42,43) but limited number of reports (44,45) indicate that serum primidone concentrations should be maintained at 5-12  $\mu$ g/ml to adequately control seizures and minimize the risk of adverse effects.

Kuh (44) reported a range of 5-10  $\mu$ g/ml although the optimal plasma or serum concentration for some individuals may vary outside this range. Most of the anticonvulsant activity of primidone rests with phenobarbital and therefore measuring phenobarbital will give the best estimate of the drug's effect (42).

Concentrations of 15  $\mu$ g/ml of primidone with therapeutic concentrations of phenobarbital have been reported to be associated with ataxia and/or somnolence (42,46,47).

Primidone is readily absorbed from the gastrointestinal tract and approximately 70-80% of an oral dose is reported to be absorbed (45). Average peak levels are reported to occur at about 3 hours, with a range of 0.5-9 hours, following single PO doses of 500 mg (48). Following oral administration of primidone, peak concentrations of primidone occur within 0.5-5 hours with

concentrations of the metabolite, phenylethylmalonamide, occurring within 8-12 hours (49,50). Bioavailability differences between two lots of primidone have been reported in the literature (51) with significant variations and serum/blood concentrations. However, Borst and Lockwood (52) observed no significant differences between two tablet brands of primidone with respect to plasma levels and seizure control. In healthy volunteers given 500 mg primidone in the fasting state the half-life of primidone was 10 to 12 hours. In patients taking primidone but no barbiturate the mean primidone serum concentration was 9.2  $\mu\text{g/ml}$  and the mean serum phenobarbital concentration was 31  $\mu\text{g/ml}$ . Primidone serum concentrations increased progressively from a mean of 32 to a mean of 14.7  $\mu\text{g/ml}$  with increasing daily doses from 0.25 to 1.25 g (48).

Pharmacokinetic studies with children (53) indicated that the plasma half-life of primidone does not differ from that in adults.

## 5.2 Distribution

Whereas phenobarbital is fairly extensively bound to plasma proteins, primidone and its other metabolite, phenylethylmalonamide, are negligibly bound. Hvidberg and Dam (42) reported primidone protein binding to be less than 20%. Others (50) presented data reporting that primidone and phenylethylmalonamide being not significantly protein bound.

Primidone and its metabolite, phenobarbital, cross into the cerebrospinal fluid space (54, 55). Vajda *et al* (56) reported significant correlation between brain and plasma concentrations of primidone in patients with epilepsy undergoing lobectomy.

Primidone readily crosses the placental barrier. Following 250 mg doses, cord blood levels were reported (57) to reach levels as high as 8.3  $\mu\text{g/ml}$  and the drug appears to be concentrated in the liver and brain of the fetus. Decreased blood concentrations of primidone and phenobarbital were noted during pregnancy in a woman receiving primidone. The blood concentrations rose again after delivery (58). Phenobarbital, its metabolite, also rapidly cross the placental barrier (58-60). Despite the rapid placental passage and high concentration that primidone and phenobarbital can reach in the embryo, they

do not appear to significantly impair prenatal development (61). In a pregnant woman receiving primidone and carbamazepine both drugs achieved significant concentrations in the breast milk (58). Phenobarbital levels were also measured. Table 9 gives serum and corresponding milk concentrations (58). Calculated levels in milk as a percentage of maternal serum concentration are: carbamazepine 63.7%, primidone 106.9% and phenobarbital 56.9%.

Table 9. Serum and breast milk concentrations of carbamazepine, primidone and phenobarbital.

Time	Source	Carbama- zine (4 to 12 mg/L)	Primidone (4 to 8 mg/L)	Phenobarbital (10 to 30 mg/L)
5 Weeks post- partum	Serum (mater- nal)	5.8	18.8	39.7
5 Weeks post- partum	Breast milk (skim fraction)	23.3	9.1	11.2
5 Weeks post- partum	Breat milk (lipid fraction)	1.4	11.0	11.4

The authors estimate that a 4 kg infant ingesting one litre of milk daily would receive about 9 mg of primidone, or 2 mg/kg/day compared with 20 mg/kg/day in the mother. Due to the multiple metabolites of primidone, milk concentrations of the drug have not been thoroughly evaluated (62). In a few reports (57,60), however, the high relative milk to serum concentrations were verified. In patients with a mean primidone serum concentrations of  $2.3 \pm 2.5$   $\mu\text{g/ml}$ , milk concentration was  $2.3 \pm 2.2$   $\mu\text{g/ml}$  or  $80.9 \pm 17.6\%$ . The phenobarbital ratio was  $45.9 \pm 24.9\%$ . It should be noted, however, that these levels reflect those of patients receiving phenobarbital, as well as those on primidone. Phenobarbital itself has previously been reported to be non-detectable in women receiving anticonvulsant doses, and to lead to excessive sleepiness in women taking hypnotic doses (60).

In one case report (60), phenobarbital-induced sedation was implicated in the death of an infant nursing from a mother treated with phenobarbital. The infant's blood level was 8.3  $\mu\text{g/ml}$ . These studies indicate that primidone levels in breast milk is present in nearly equal concentration to that of the maternal serum. Phenobarbital is present in about 50%. The apparent volume of distribution is reported to be 0.6 L/Kg (42).

### 5.3 Metabolism

Primidone is metabolized by the liver in man, dog, rabbit, rat and mouse to two major metabolites, phenylethylmalonamide (PEMA) (42,49, 63-66) and phenobarbital (49, 65-73). Both PEMA and phenobarbital possess anticonvulsant activity; however, PEMA has only weak anticonvulsant properties and is more toxic than primidone. Approximately 15-25% of an oral dose of the drug is metabolized to phenobarbital and about 50-70% metabolized to PEMA (45). Phenobarbital is reported to appear in plasma following 4 days continuous primidone therapy (72,73). p-Hydroxyphenobarbital and its glucuronide conjugate are also reported (64,66,70) as minor metabolites of primidone. Recently, Haidukewych and Rodin (74) monitored PEMA in serum by GLC and applied the technique for retrospective study in epilepsy patients dosed with primidone.

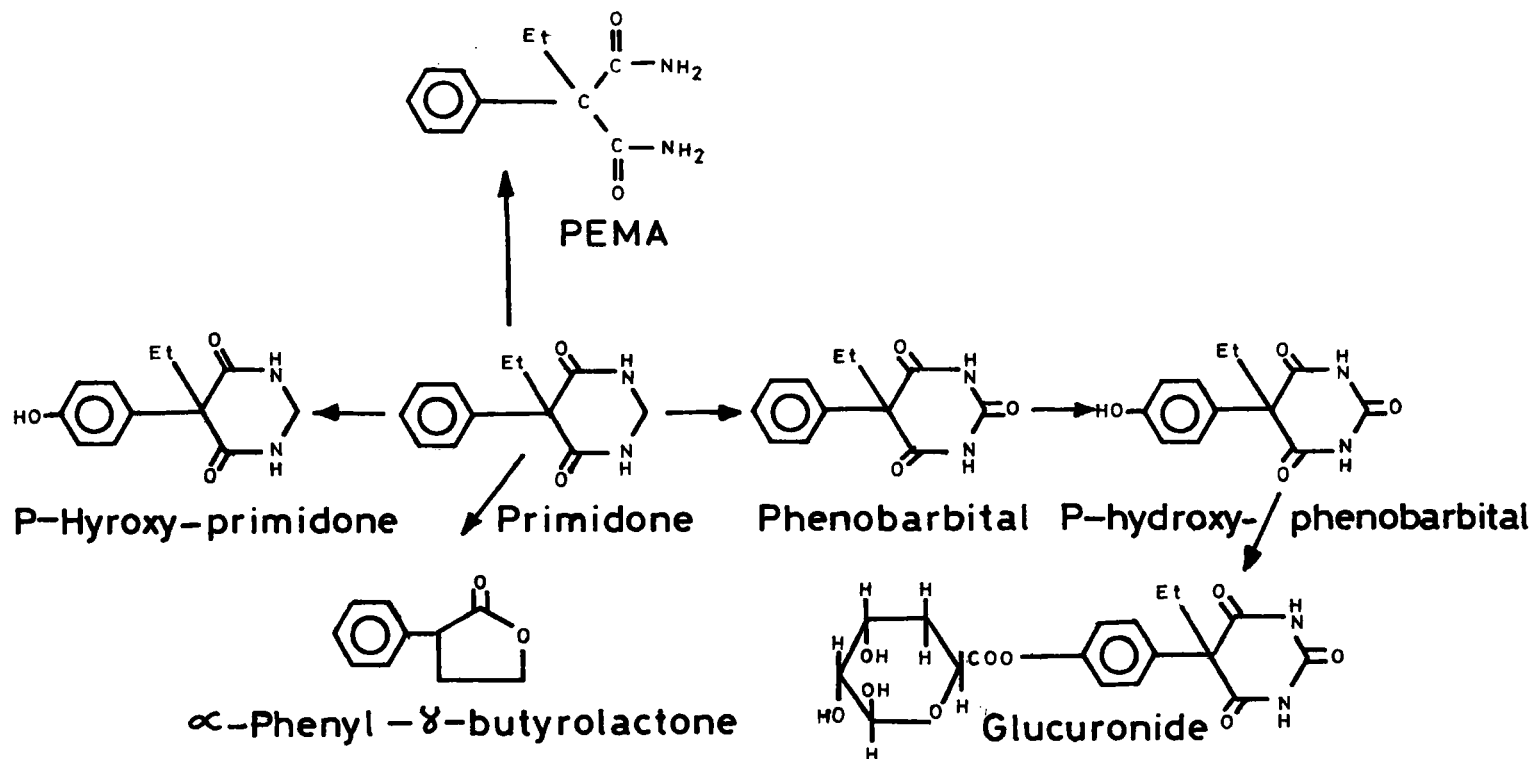
Andresen *et al* (75) isolated a novel metabolite,  $\alpha$ -phenyl- $\gamma$ -butyrolactone, in urine samples of patients severely intoxicated with primidone, phenobarbital or glutethimide.

p-Hydroxyprimidone was recently identified as a minor metabolite of primidone in rat and man by Hooper *et al* (76). Analysis of urine specimens from rats and humans after a single dose of primidone, revealed in addition to the previously documented metabolites of primidone, a small peak which was shown to be due p-hydroxyprimidone as a new minor metabolite of the drug.

The chemical structures of the metabolites are shown in Scheme 2.

### 5.4 Elimination

Primidone is metabolized in the liver and excreted in the urine as phenylethylmalonamide, phenobarbital and p-hydroxyphenobarbital. Primidone has a relatively short



Scheme 2 : Metabolism of primidone

plasma half-life of about 10 hours (42,50) compared with those of its principal metabolites, phenylethylmalonamide with plasma half-life in the region of 29-36 hours (50) and phenobarbital whose estimated plasma half-life ranges from 48-84 hours (49). Almost no primidone is excreted unchanged in urine (42) although phenobarbital is found in urine in a percentage of 15% of the dose (66). However, during chronic therapy with primidone (45) approximately 15-25% of an oral dose is excreted in urine as unchanged drug, 15-25% is metabolized to phenobarbital and 50-70% is excreted in urine as phenylethylmalonamide. Primidone is removed by dialysis.

Primidone crosses the placental barrier and is excreted in milk. The amounts of primidone actually consumed by a nursing infant are very small, and in most cases, pharmacologically insignificant, although sedation may occur (See also Distribution).

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## ERRATA

CIMETIDINE—Volume 13, p. 127

The following references inadvertently had been omitted from p. 182:

<sup>35</sup> Deleted.

<sup>36</sup> Deleted.

<sup>37</sup> Lofton, F., Smith Kline & French Laboratories, personal communication.

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